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PREFACE

It is interesting to reflect upon the future of scientific publications. The second edition of the *World List*, published in 1934, listed 36,000 scientific periodicals. Fifteen thousand of these were classified by Bradford as useful, the remainder as containing papers of less importance. The 15,000 were publishing in 1934 at the rate of 750,000 good or fair papers per year. According to E. W. Hulme the yearly average of papers in the pure sciences was close to 3,100 for the period 1800-1863, rising to about 22,600 for the period 1874-1900 and to 85,500 in 1910. The implications of these data are sobering, not only because of the perplexities that now confront the reader, the publisher, and the librarian, but because of the exponential nature of the growth of scientific literature: future generations will find themselves buried in paper and entombed in libraries unless paper pulp reserves run out or some sensible solution to the problem is found.

The abstracting and reviewing services in the sciences obviously call for study. The abstracting services, guilty incidentally of much duplication of effort, have greatly increased, but their coverage of "good" and "very fair" papers shortly before the war barely exceeded one third of the 750,000 original papers.

The effects of the Six-Year War on scientific publications have escaped a satisfactory appraisal but, despite the unfortunate demise of some journals, we would be greatly surprised if the world totals of papers and journals for 1948 were not considerably greater than those for 1934. While the consumers of this inundating flood of papers have increased, the number of journals awaiting perusal by a diligent reader in any of the sciences has increased—so also the abstracts over which he must pore if his avidity is still unquenchable.

It has been our hope that in these *Annual Reviews* our colleagues in Biochemistry, Physiology, and Microbiology would receive some relief through a sort of "predigestion" process. The increases in papers and in journals, however, have imposed their problems upon the reviewer and the publisher as well. The reader has been asked, even from the first, to accept critical appraisals of the subjects of his interest rather than comprehensive reviews of the synoptic type. The reviewers have been asked by our editors to screen the literature even more severely, if need be to review but a fraction of the papers of the preceding year or biennium. Recent volumes

of the *Annual Review of Biochemistry* have approached or exceeded eight hundred pages. In order that the *Review* may not become even more unwieldy in size, the Board of Directors have considered two possible solutions, neither of which is without its disadvantages. The allocations of space could be made even more rigorous and severe, or a portion of the subject matter, rather cleanly contained in a few of the chapters, could be transferred to a new *Review*. The latter, after, an exhaustive study of the problem, has been decided upon as the more adequate solution. In 1950 the Board propose to publish the introductory volume of an *Annual Review of Plant Physiology*. In so doing it will be the hope of the Editor and the Editorial Committee that there may be made available to their colleagues in plant physiology and plant nutrition systematic annual reviews of the literature that will be fully worthwhile.

Indeed a wide study of the problem of *Reviews*, in so far as other sciences are involved, has convinced the Board that their seventeen-year experiment with the *Annual Review of Biochemistry* almost requires an extension of the project into still other sciences. So it is that the organization of an *Annual Review of Psychology*, an *Annual Review of Physical Chemistry* and an *Annual Review of Medicine* has been recently announced, with Volume I of each scheduled to appear in 1950.

In presenting this, the seventeenth volume of the *Annual Review of Biochemistry*, to our colleagues we do so with a deep sense of gratitude to those who have served as its authors. We regret that Dr. Hugh Sinclair's review on Nutrition, almost ready for the post, was lost in Europe with other papers and personal effects. We are grateful to Professor G. W. Beadle for permission to reprint his review on Genetics from Volume X of the *Annual Review of Physiology*.

Our very sincere thanks are extended to our editorial assistants who helped so greatly in preparing this volume for the press, and to our printers, the George Banta Publishing Company, for their continued cordial collaboration.

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Volume XVI

page 1, line 16: *for l-malonate read l-Malate*

page 203, line 5: *add (159).*

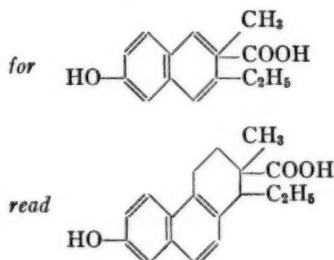
page 207, line 22: *for to a fraction read to a substance postulated to be present in a fraction.*

page 222: *add to bibliography 159. SLINGER, S. J., MACIL-RAITH, J. J., AND EVANS, E. V., Poultry Sci., 25, 628-41 (1946)*

page 260, line 34: *for carpylate read caprylate*

page 271, Ref. 232: *for Am. Scientist, 24 read Am. Scientist, 34*

page 298, Formula VI:



page 371, line 22: *for last read later*

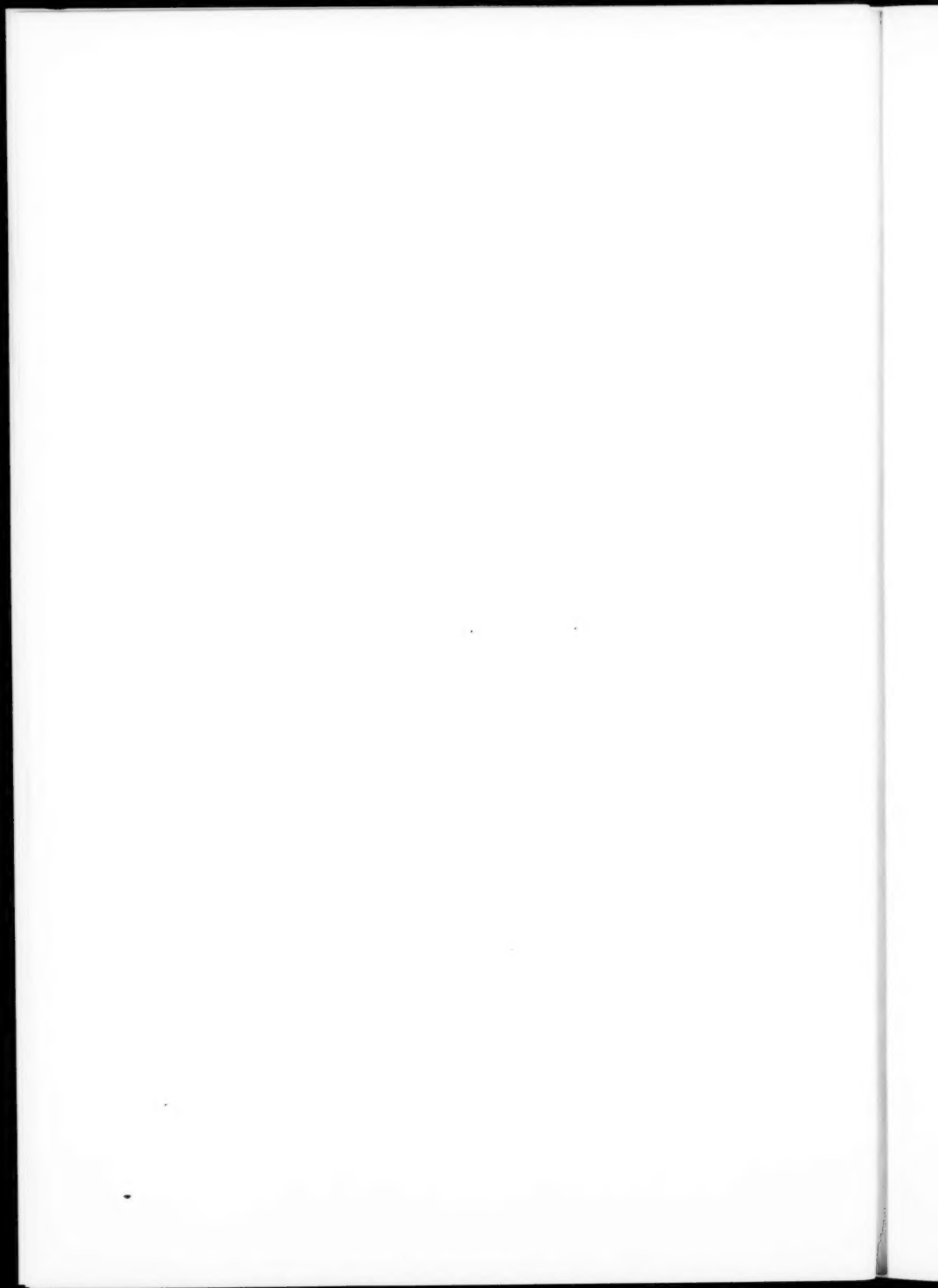
page 371, line 23: *for immediate read intermediate*

page 371, line 28: *for dimethylethanolamine read mono-methylethanolamine*

page 424, line 14: *for 1 mg. read 1 µg.*

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BIOLOGICAL OXIDATIONS AND REDUCTIONS¹

BY H. WEIL-MALHERBE

The Laboratories, Runwell Hospital, Wickford, Essex

OXIDATION CATALYSTS

CYTOCHROME AND CYTOCHROME OXIDASE

The oxidation-reduction potential of cytochrome-*c* has been studied by Paul (1) and by Rodkey & Ball (2). Whereas previous investigations were confined to a narrow pH range around 7, these new measurements extend over a much wider range, designed to reveal any dissociation constants due to heme-linked groups in the molecule. The results are in fairly good agreement, though differing in detail. Ferricytochrome-*c* has a dissociation constant of nearly 10^{-7} [$pK = 6.86$ according to Paul (1); 7.70 according to Rodkey & Ball (2)]. The $E_0' = pH$ curve has a slope of 0.0 between pH 1.75 and 7.70 ($E_0' = +0.254$ v.) and of 0.060 between pH 7.70 and 10.0. There is some indication that below pH 1.75 the slope is 0.120 (2). Light absorption measurements reveal for ferrocytochrome-*c* a pK value of 9.28 (1).

Azide, like cyanide (3), forms a well-defined complex with ferricytochrome-*c*, though not with the ferrous form (4). The compound is characterized by a shift of the absorption maximum from 5300 Å to 5400 Å, by the appearance of a faint new band at 5750 Å and, again in analogy to the cyanide compound, by the disappearance of the band at 6925 Å. The azide complex differs from the cyanide complex by the fact that it is highly dissociated and that its formation is almost instantaneous. Whereas cytochrome-*c*, like methemoglobin, reacts only with the cyanide or azide ions, cytochrome oxidase combines with the undissociated acids. This conclusion is based on an analysis of the inhibition as a function of pH (5).

Michel & Scheinberg (6) did not observe any beneficial effects of intravenous injections of cytochrome-*c* on anoxic or cyanide-poisoned rats, as claimed by Proger *et al.* (7). Since Albaum *et al.* (8) had shown that after cyanide poisoning 50 per cent of rat brain cytochrome oxidase was still functional, an increased supply of cytochrome-*c* might conceivably have increased the efficiency of

¹ This review covers the period from October, 1946 to September, 1947.

the uninhibited fraction. However, intravenous injection of cytochrome-*c* did not prolong the survival time of rats in an anoxic atmosphere nor had it any effect on biochemical changes produced by cyanide poisoning or anoxia, such as the lowering of tissue pyrophosphate or the increase of blood lactate.

Warburg (9) has computed the molecular weight of what he still calls "the oxygen transferring ferment of respiration" as 75,000 per mole hemin. This calculation is based on the observation of Bücher & Kaspers (10) that the quantum yield of the photochemical dissociation of carbon monoxide myoglobin is independent of the wave length of the irradiating light, even at 2800 Å where 40 per cent of the total absorption is due to the tyrosine and tryptophane residues of the protein component, indicating that light absorbed by the protein component has the same photochemical efficiency as light absorbed by the hemin component. This fact can be used to arrive at the molar absorption coefficient of the protein component of the enzyme.

In mouse skin painted with methylcholanthrene, cytochrome oxidase activity increases gradually during the hyperplastic stage and reaches twice its normal value in the final stages (11). In the resulting squamous carcinomas the activity is less than in late hyperplasia, but still greater than in normal epidermis. Succinic dehydrogenase activity, too, is four times higher in squamous carcinoma than in normal epidermis. This is in striking contrast to the decrease of these enzymes in many tumors of different origin.

A nonrespiratory variant of *Saccharomyces cerevisiae* which is characterized by the absence of cytochromes-*a* and *b* and of cytochrome oxidase has been described by Whelton & Phaff (12). It contained, however, cytochrome-*c*, thus differing from Castor's cyanide yeast which was lacking in this component as well.

CATALASE

With the aid of his elegant rapid flow method [cf. (13)] Chance (14) has revealed the existence of an intermediate enzyme-substrate compound in the reaction of catalase and hydrogen peroxide. The dissociation of the complex is, however, so slow that its direct participation in the catalase reaction is unlikely. The formation of this compound probably does not involve each of the four hemin groups of the catalase molecule to the same extent, but the reactivity of any free hemin group may well be affected by the com-

pound. On the other hand, there are reasons to believe that the enzyme-substrate complex is responsible for the peroxidatic oxidation of alcohols (211) since its decomposition is accelerated by lower alcohols. That the different hemin groups of catalase are not equivalent is a conclusion arrived at independently by other authors. George (15) has published a penetrating study of catalase kinetics which confirms many of Chance's deductions. He finds that the reaction between catalase and hydrogen peroxide consists of two phases: a rapid initial reaction, decreasing exponentially with time and practically terminated after two minutes, and a superimposed slower, steady reaction. These two phases are assumed to be due to the fact that, in the presence of hydrogen peroxide, catalase can exist in two forms, a labile, more active form and a stable, less active form. The former changes reversibly to the latter during the initial phase. The reaction rate of both phases is accelerated by increasing substrate concentration up to a maximum, which is not identical for the two, and is reversibly inhibited by higher substrate concentrations. This inhibition is due to the formation of an enzyme-substrate complex of low catalytic activity. The transition from the initial rapid rate to the steady rate can be explained by the setting up of an equilibrium between the enzyme-substrate complex and its decomposition products. This is represented by a reaction in which the enzyme-substrate complex EP dissociates into E' and reaction products and another reaction in which the enzyme in its original form E is regenerated by the substrate P : $E' + P \rightarrow E + \text{reaction products}$. If, as George suggests, E and E' represent ferric and ferrous forms of catalase, the labile, more active form of catalase may be identified with an equilibrium mixture of the ferric enzyme and its substrate complex and the less active form with an equilibrium mixture of peroxide complex, ferric enzyme and ferrous enzyme. It will be noticed that George postulates a reoxidation of ferrous catalase by hydrogen peroxide, in accordance with the theory of Weiss (16).

According to Agner & Theorell (17) the iron atoms of catalase are combined with hydroxyl groups which may be displaced not only by well-known catalase inhibitors, but probably by all, at least low-molecular, anions in sufficient concentration. This causes inhibition of activity in a corresponding degree. Formate is a particularly strong inhibitor, whereas the results with acetate are anomalous and lead to the conclusion that the different hemin

groups of catalase have different properties both spectrophotometrically and with respect to their individual contributions to the total activity. Anion inhibition increases with decreasing pH. The true pH-effect on catalase is much smaller than was previously supposed, since the fall of activity in buffered solutions at lower pH-values is largely due to anion inhibition.

Although crystalline horse blood catalase contains four hemin groups and crystalline horse liver catalase three hemin and one verdohemochromogen group, they both have identical protein components (18).

Pointing out that catalase can be inhibited by both oxidizing and reducing agents Hoffmann-Ostenhof (19) suggests that this can be most simply explained by an interruption of the valency changes of catalase iron. He proposes a modification of Keilin & Hartree's theory (20) designed to meet the objections of Weiss & Weil-Malherbe (21). Hoffmann-Ostenhof seems to be under the erroneous impression that Weiss & Weil-Malherbe's criticism of Keilin & Hartree's scheme was directed against their assumption of a valency change of catalase iron. This is not the case; indeed they pointed out that a theory of catalase action embodying a valency change mechanism was first put forward by Haber & Willstätter in 1931. The bone of contention was rather the question whether the reoxidation of ferrous catalase was effected by molecular oxygen, as proposed by Keilin & Hartree, or by hydrogen peroxide, as proposed by Weiss. The principal objection to the former scheme, which has never been satisfactorily answered, was that in the course of its reduction the oxygen molecule would have to pass through the hydrogen peroxide stage and the enzyme action would thus cancel itself out. It is very doubtful if a "direct" reduction of oxygen to water, as assumed by Keilin, exists. According to modern concepts chemical reactions can be resolved into simple elementary mechanisms involving the displacement of single electrons or protons [cf. Hinshelwood (22); Michaelis (23)]. It is probable, therefore, that the reduction of oxygen to water is always a step-wise process involving the—however transitory—production of hydrogen peroxide or of a radical of a corresponding level of reduction. Since the experimental mainstay of Keilin & Hartree's scheme viz., the anaerobic inactivity of catalase, has now been withdrawn by the authors themselves (24), their theory, as far as

it implies reoxidation of ferrous catalase by oxygen, can, in the reviewer's opinion, no longer be maintained.

The very striking fall of catalase activity in the liver of tumor-bearing animals has been the subject of an investigation by Weil-Malherbe & Schade (25). They found that, in relation to the ratio of body weight to tumor weight, the liver catalase of tumor-bearing rats fell to an equal extent whether the animals were kept on a high- or on a low-protein diet, even in the initial stages of tumor development.

Serfaty (26) has written an interesting review of his work on catalase.

PEROXIDASE

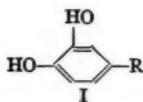
Peroxidase activity can be demonstrated in most tissues of rat and guinea pig according to DuBois & Sebesta (27). Rat lung peroxidase is inhibited by α -naphthylthiourea. Inhibition of thyroid peroxidase by thiourea is again suggested by De Robertis & Grasso (28) as an explanation of the antithyroid action of thiourea; this may result in an inhibition of the oxidation of iodide to iodine. This is improbable in view of the results of Vanderlaan & Bissell (29) which show that even when given in maximal doses over a long period thiouracil did not prevent the rapid accumulation of iodine in the thyroid after injection of potassium iodide in young chicks [see also (30)].

TYROSINASE

The tyrosinase of the insect cuticle.—The dominating part played by tyrosinase in the darkening and hardening of the insect cuticle has been further elucidated. In 1940 Pryor (31) showed that the hardening of the insect cuticle is due to a phenol, derived from the blood, which is oxidized by tyrosinase to a quinone and combines with the protein producing hardening and darkening by a tanning process. Dennell (32) has now made a detailed study of the pupation of *Sarcophaga falcitata* with the following results: the active phenol is derived from blood tyrosine and is found in the blood at the time of pupation. In the late larva tyrosine and tyrosinase co-exist in blood, but the oxidation of tyrosine is inhibited until the onset of pupation owing to the low oxidation-reduction potential prevailing in the blood of mature larvae (0.12 to 0.13 v.). The

maintenance of this low potential probably involves the activity of a dehydrogenase system which, in turn, is inhibited or destroyed at the time of pupation. Liberation of the pupation hormone from the gland known as Weismann's ring is followed by a steep rise in the oxidation-reduction potential of blood, thus releasing tyrosinase activity. The oxidation of tyrosine results in the production of an *o*-dihydroxyphenol which passes into the cuticle, accumulates in the outer endocuticle and is oxidized to the *o*-quinone at the inner epicuticle, the seat of tyrosinase. Therefore darkening proceeds inward from without, although the phenol is supplied from within. Fraenkel & Rudall (33) concluded from the *in vitro* effect of various phenols on isolated larval cuticles that the active phenol is derived from deaminated tyrosine. The dehydrogenases whose action keeps tyrosine in the reduced state are inhibited by methanol. Thus darkening and hardening of the cuticle takes place if mature larvae are placed alive in methanol. The gain in cuticle weight at pupation can be accounted for by the loss of free tyrosine from the blood. In good agreement with these facts is Heller's observation (34) that addition of codehydrogenase I to ground chrysalids of hawk moths results in stimulation and prolongation of respiration and in retardation of melanization.

Identification of certain phenols concerned in the hardening of insect membranes has been achieved by Pryor, Russell & Todd (35, 36). They isolated three different substances which can all be regarded as derived from 3,4-dihydroxyphenylalanine by deamination and, eventually, decarboxylation and which possess the structure given in Formula I.

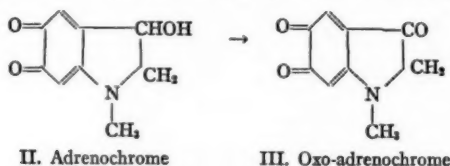


Protocatechuic acid ($R = \text{COOH}$) was shown to be present in the cockroach ootheca and in blowfly puparia, whereas adults of *Tenebrio* contained 3,4-dihydroxyphenylacetic and -lactic acids ($R = \text{CH}_2\text{COOH}$ and $\text{CH}_2\cdot\text{CHOH}\cdot\text{COOH}$).

That the processes described are not confined to the insect world alone is shown by the work of Ellenby (37) who demonstrated the presence of active tyrosinase in the cyst wall of the potato-root eelworm, a nematode, and its participation in the

darkening and hardening of the membrane. The presence of tyrosinase in the cuticle of crustaceans at the time of moulting is reported by Dennell (38). Since there is also a tyrosinase system in crustacean blood [Pinhey (214)], the situation seems to be very similar to that in insects.

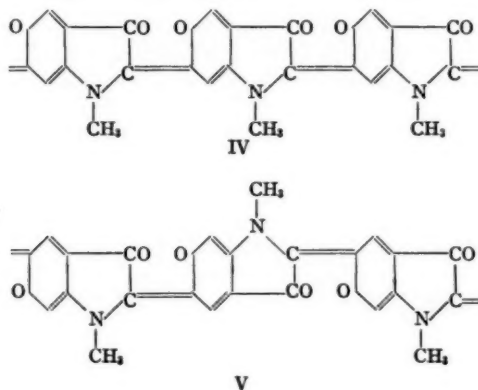
The action of tyrosinase on proteins.—Sizer's claim that mushroom tyrosinase may oxidize protein-bound tyrosine (39) has been criticized by Edman (40) who came to the conclusion that the effects were due to free tyrosine present as a result of contamination or autolysis. On reinvestigation, for which he used rigorously purified proteins, Sizer (41) admitted that autolysis products were responsible for a fraction of the effects, but maintained that they could not entirely account for them and that oxidation of tyrosyl groups actually occurred in the intact protein. The question of the activity of proteases acted on by tyrosinase was left open. According to Drouineau & Gouny (42) the instability of catalase in



plant press juices is due to oxidation by tyrosinase: catalase can be protected by low concentrations of cyanide.

The constitution of melanin.—There has been a revival of interest in the chemistry of melanin. Clemo & Weiss (43) suggested that 5,6,5',6'-tetrahydroxyindigo, possibly in the form of the corresponding quinone or semiquinone, is the principal constituent of natural melanine. This hypothesis is supported by the known facts of the mechanism of tyrosine oxidation, by the oxygen consumption of the process and by the nitrogen content of the purest specimens of melanin. They failed, however, in their efforts to prepare tetrahydroxyindigo. This has now been achieved by Harley-Mason (44), but the properties of the substance proved to be quite different from those of natural melanin. Certain discrepancies between the analytical figures of melanin and those required for a substance composed of tetrahydroxyindigo units were pointed out by Burton (45). Perhaps the most significant contribution to the problem has come from Cohen (46). He showed that adrenochrome

II, known to be an intermediate in the melanization of adrenalin, is first oxidized to oxo-adrenochrome III. Like other indoxyls, oxo-adrenochrome forms indogenides by condensation with aromatic aldehydes or with ketones. By polymerization, chains of either the indigo IV or the indirubin V type of linkage are formed. The structure of natural melanin is assumed to correspond to the



indirubin type on the basis of analogy with the behavior of other compounds of this class and because the fluorescence spectrum of reduced melanin is very similar to that of leuco-indirubin and quite unlike that of leuco-indigo.

During the melanization of tyrosine, catalyzed by a tyrosinase from transplantable mouse melanoma, hallachrome is formed as an intermediate (47). The action of this mammalian tyrosinase is therefore presumably the same as that of tyrosinase from other sources.

The presence of a tyrosinase inhibitor in the serum of cancer patients has been claimed by Duboff & Hirschfeld (48) and denied by Stadie, Perlmutter & Robinson (49).

LACCASE

Bertrand (50, 51, 52) has continued his studies on laccase [cf. (53)]. The enzyme oxidizes reduced cytochrome-*c* and cysteine and thus resembles cytochrome oxidase, but whereas cytochrome oxidase oxidizes *p*-phenylenediamine six times faster than hydroquinone, the rate is about the same for both in the case of laccase.

FLAVOPROTEINS

Phosphate extracts of dried pig kidney cortex were found by Iselin & Zeller (54) to oxidize L-phenyllactic, L-leucinic, L-iso-leucinic and DL-mandelic acids to the corresponding keto acids, but to be inactive towards L-amino acids. The authors nevertheless consider this enzyme to be identical with Green's L-amino acid oxidase because the specificity towards hydroxy acids, the distribution in animal tissues, the action of inhibitors and the inactivity of pyridine nucleotides are similar for both enzymes. The inactivity of the pig enzyme towards L-amino acids is interpreted as a variation due to difference of origin.

A similar enzyme seems to occur in various strains of tubercle bacilli and other acid-fast bacteria, since these were shown by Roulet, Wydler & Zeller (55) to oxidize a similar set of L-hydroxy acids. The enzyme in question may be identical with that isolated by Edson (56) from *Mycobacterium phlei*, which is indeed a flavoprotein containing flavin-adenine-dinucleotide as prosthetic group. The enzyme oxidizes L-lactate to pyruvate and hydrogen peroxide. Though it may be closely related to Green's L-amino acid oxidase, the latter enzyme contains riboflavin phosphate as prosthetic group.

The quinine-oxidizing flavoprotein from rabbit liver [cf. (57)] oxidizes N-methylnicotinamide to the 6-pyridone which appears in human urine and accounts for about 10 per cent of administered nicotinamide (58).

Flavoproteins, in particular a purified preparation of Straub's flavoprotein from heart, were found to act as hydrogen carriers in the enzymatic reduction of trinitrotoluene to 4-amino-2,6-dinitrotoluene (59).

According to Polonovski *et al.* (60) the activity of xanthine dehydrogenase in fresh milk is very weak so long as the temperature does not fall below 20°C. On passing from 20° to 15° there is an abrupt rise of activity to a new level which remains constant at temperatures below 15°C. The critical temperature corresponds to the solidification point of milk fat. It is assumed that the enzyme is secreted in the form of a protein-lipid complex and that on cooling irreversible dissociation takes place. Dissociation can also be induced by detergents without cooling. A steady rise of xanthine oxidase activity associated with the growth of yellow fever virus in mouse brain has been reported by Bauer (61). It would be

interesting to compare the xanthine oxidase content of mouse milk from strains which do, and from those which do not, possess Bittner's tumor-inciting "milk factor."

The prosthetic group of notatin, the glucose oxidase of *Penicillium notatum* (62, 63), has now been identified with flavin-adenine-dinucleotide by Keilin & Hartree (64).

Edlbacher *et al.* (65, 66, 67) studied the effects of L-amino acids on the activity of D-amino acid oxidase. With high concentrations of enzyme inhibition of a competitive type occurs. But with low concentrations of enzyme, and especially if highly purified enzyme is used, addition of amino acids of either the L- or the D-series causes an activation. The greatest effects are obtained with L- or D-histidine which acts even in peptide linkage and which, in concentrations of a few $\mu\text{g. per ml.}$, may increase enzyme activity twentyfold. In view of the well-known propensity of histidine to form heavy-metal complexes and, moreover, in view of the fact that similar effects are obtained with cyanide or pyrophosphate there can hardly be any doubt that the activation is largely due to a removal of heavy-metal impurities.

Kojic acid competitively inhibits D-amino acid oxidase (68).

DEHYDROGENASES

Weinmann, Morehouse & Winzler (69) investigated the effect of succinic dehydrogenase on α, α' -dideuterosuccinate in absence of hydrogen acceptors. They found that there was a definite exchange between the deuterium of succinate and the hydrogen of water molecules even in absence of the enzyme. In its presence the rate of exchange was increased to an extent corresponding to the rate of succinate oxidation of which the enzyme was capable. Succinic dehydrogenase therefore catalyzes two reactions: (a) a transfer of hydrogen from succinate to an appropriate acceptor, (b) an exchange between the α -hydrogen of succinate and the hydrogen ions of the medium. Whether the dissociation of the substrate hydrogen into proton and electron occurs directly from an enzyme-substrate complex, or indirectly after transfer to a reducible group of the dehydrogenase itself or of another hydrogen acceptor, could not be decided; the first alternative was favored as being in accord with the theory of Potter & DuBois in which the enzyme function is visualized as an oscillation between the normal thiol form and its free radical, with electrons passing to the prosthetic group of the

enzyme and hydrogen ions passing into the solution. Farkas *et al.* (70, 71) came to very similar conclusions after studying the bacterial hydrogenation of fumarate by atmospheric hydrogen containing an admixture of deuterium, only here the issue is further complicated by a second enzymatic exchange reaction between gaseous hydrogen and water.

Stoppani (72) separated, by ammonium sulfate fractionation of liver extract, succinic dehydrogenase and a factor which links this to the cytochrome system. Without this factor the dehydrogenase reacts only slowly with cytochrome-*c* or methylene blue, though rapidly with ferricyanide. The factor is apparently not required by α -glycerophosphoric dehydrogenase, since muscle extract without the factor, which is unable to oxidize succinate, oxidizes α -glycerophosphate in presence of cytochrome-*c*.

The liver succinic dehydrogenase is significantly decreased in riboflavin-deficient rats with little change in cytochrome oxidase concentration, according to Tipton (73). Elson (74), on the other hand, finds that deficiency in B-vitamins lowers the cytochrome-*c* concentration in liver while succinic dehydrogenase and cytochrome oxidase remain relatively unaffected. A low-protein diet mainly reduces the dehydrogenase component of the succinoxidase system.

The succinoxidase content of rat hepatomas produced by feeding 2-acetaminofluorene is about the same as that of normal rat liver (75), but greatly reduced in rat hepatomas produced by feeding *p*-dimethylaminoazobenzene (76).

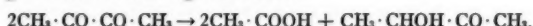
Some years ago Houchin (77) claimed that succinoxidase activity was increased in muscles of dystrophic hamsters and reduced to its normal level by addition *in vitro* of tocopheryl phosphate. An inhibition of the succinoxidase of normal muscle by tocopheryl phosphate was later reported by Govier *et al.* (78). Basinski & Hummel (79) have now repeated Houchin's experiments without confirming them. They found that the succinic dehydrogenase activity and its inhibition by tocopheryl phosphate were the same in normal and in dystrophic hamster muscle. Ames (80) drew attention to the insolubility of calcium tocopheryl phosphate. He showed that addition of calcium chloride relieved the inhibition and that the amount of tocopheryl phosphate required to achieve the same degree of inhibition was proportional to the calcium concentration. The removal of calcium ions from the solu-

tion results in better preservation of codehydrogenase I and consequently in formation of the strongly inhibitory oxaloacetic acid by malic dehydrogenase. Thus it appears that tocopheryl phosphate removes the calcium that activates the nucleotidase that destroys the coenzyme that catalyzes the oxidation of malate to oxaloacetate,—the substance that inhibits succinic dehydrogenase. L- but not D-glutamate partially relieves the inhibition caused by tocopheryl phosphate by removing oxaloacetate through transamination, whereas addition of codehydrogenase I greatly enhances the inhibition.

Ames' interpretation of the increased respiration observed in dystrophic muscle is less plausible. This will be further discussed below (p. 23).

THIAMINE ENZYMES

Diacetyl mutase is the name given by Green, Stumpf & Zarudnaya (81) to an enzyme widely distributed in animal tissues which catalyzes the dismutation of diacetyl to acetoin and acetic acid according to the following reaction:



The enzyme contains cocarboxylase as prosthetic group; it can be split by acidification to pH 4.

The preparation of pyruvic and α -ketoglutaric oxidases from pigeon breast muscle by Stumpf, Zarudnaya & Green (82) represents a great advance. The same particle suspension used for the study of diacetyl mutase was found to catalyze the oxidative decarboxylation of the two α -keto acids, though there is evidence to show that the enzymes responsible for the oxidation of pyruvate and ketoglutarate are not identical. A nonoxidative decarboxylation, low at pH 8, occurs as a side reaction [cf. (83)]. Cocarboxylase, acting as a prosthetic group, can be split off from the enzymes by mild acidification, but the ease of dissociation is different for the two enzymes. Cocarboxylase is the only component necessary for the activity of the enzymes. No inorganic phosphate is required and no evidence was found for the formation of acetyl phosphate. Phosphate and other activators previously reported to be essential for pyruvate oxidation, such as ATP, flavoproteins, dibasic acids, must be assumed to intervene in later stages of the process. The pyruvic enzyme is not inhibited by iodoacetate. This is in agreement with the statement (105) that the essential thiol group of the

pyruvic oxidase system is not located in the pyruvic dehydrogenase component.

UNCLASSIFIED OXIDASES

Soybean lipoxidase has now been crystallized by Theorell, Holman & Åkeson (84). The enzyme is apparently a simple protein of molecular weight 90,000 to 100,000 without recognizable prosthetic group or heavy-metal association. One mole of enzyme produces about 290 moles of peroxide per sec. at 20°. Lipoxidase action and autoxidation of fatty acids lead to very similar products according to Holman (85) [cf. (86)].

The oxidation of reduced glutathione by tissue extracts is not due to cytochrome oxidase and is not preceded by hydrolysis of the substrate. The oxidase is most active in mouse kidney (87).

The specificity of amine oxidase towards a number of amines was investigated by Randall (88). He found oxidation to be slower with tertiary than with primary or secondary amines whereas quaternary salts were not attacked at all. Substitution in the benzene ring markedly influenced the relative rates of oxidation. There was no close correlation between the order of oxidizability and the intensity or duration of sympathomimetic action.

Oxidative deamination of L-phenylalanine, L-tyrosine and L-tryptophane has been shown to occur in bull spermatozoa by Tosič (89). The reaction is accompanied by hydrogen peroxide production.

CODEHYDROGENASES

As Mehler *et al.* (90) report, the specificity of dehydrogenases towards codehydrogenases I and II ranges from complete interchangeability in the case of L-glutamic dehydrogenase to complete specificity for codehydrogenase II in the case of isocitric dehydrogenase. Malic dehydrogenase reacts fifteen times faster and lactic dehydrogenase one hundred times faster with codehydrogenase I than with II.

An observation of unusual interest is announced by Raska (91) who produced severe, lethal pellagra in dogs by giving them 400 mg. doses of adenine by mouth. On the fourth day the first symptoms had already appeared. An interference by adenine in the formation or utilization of codehydrogenases may be assumed.

A number of papers have appeared giving improved methods of preparation or estimation of codehydrogenase I (92 to 96).

Cocarboxylase.—The possibility of cocarboxylase acting as part of a reversible oxidation-reduction system has again been considered. Whereas in Lipmann's scheme a reduction of the pentavalent thiazol nitrogen was envisaged, a second possibility was opened by the discovery of the thiol form of thiamine and its oxidation to the disulfide by Zima & Williams (215). Theoretically there are thus two possible redox systems in one of which cocarboxylase would be in the oxidized and in the other in the reduced form. The disulfide form of cocarboxylase was prepared by Karrer & Visconti (97) and was found to be inactive in the test with washed yeast. This was confirmed by Peters (98) who found, however, that thiamine-disulfide, and probably cocarboxylase-disulfide too, were fully active in the catatorulin test with deficient pigeon brain. Since decarboxylation is oxidative in animal tissues and nonoxidative in yeast, this result may indicate a significant difference of mechanism, though it may simply mean that brain tissue, but not washed yeast, can reactivate the disulfide by reduction. Investigation of the possible catalytic role of the system thiamine-dihydrothiamine has so far been hampered by failure to isolate the reduced component. Reduction of thiamine with sodium dithionite only yields an inactive brown gum. Not unexpectedly, the reaction has now been shown by Karrer *et al.* (99) to lead to a reductive cleavage of the molecule. But their conclusion that this result rules out the existence of the system thiamine-dihydrothiamine seems hardly justified. The splitting of the thiamine molecule by sulfonating agents such as sulfite is well known and the action of dithionite may be related to this phenomenon rather than to the reduction as such. The possibility of a reduction without cleavage by other agents cannot be excluded. Such a possibility is suggested by the experiments of Ågren (100) who showed that a reducing phenol isolated from aspen leaves, and also cysteine and ascorbic acid, change thiamin in such a way that it no longer reacts with the diazo reagent while still giving an undiminished thiochrome reaction. Isolation of the reduction product would be highly desirable.

THIOL GROUPS

The biochemistry of BAL.—A spate of some thirty papers dealing with the action of 2,3-dimercaptopropanol (British Anti-Lewisite, BAL) and related compounds has appeared during the year. It is impossible to do full justice to this important work in

the space available and not more than a very short synopsis can be given. The essential facts have previously been summarized by Elliott (101), and Peters has recently written a more extensive review (102) of the subject.

The toxic action of many metals and metalloids is due to their combination with essential thiol groups followed by inhibition of enzyme action. The mercaptides formed may be caused to dissociate by the addition of other thiol compounds which give rise to mercaptides of greater stability. An analysis of the reaction product of lewisite with kerateine (103) showed that thiol groups which disappeared far exceeded the arsenic atoms bound and that at least 75 per cent of the arsenic was combined with two thiol groups, thus forming rings of great stability. The superiority of BAL as an arsenical antidote over other dithiols has been correlated with the greater stability of the six-membered ring formed by the reaction with arsenic, whereas the relative inefficiency of monothiols may be attributed to the low stability of open chain mercaptides (104).

Among several enzymes examined the pyruvic oxidase system of brain proved to be the most sensitive to poisoning by lewisite and arsenite (105). Inhibition of the system by maleic acid and by disulfide groups provides evidence for the presence of an essential thiol group (106). Succinic dehydrogenase, though also a sulfhydryl, was less sensitive; at low concentrations of the arsenical the poisoning of the pyruvic oxidase system is therefore specific. The total oxidase is more sensitive than the pyruvic dehydrogenase component (105). Pyruvate oxidation in skin is equally inhibited by lewisite (107). BAL protects brain pyruvic oxidase (108) and succinic oxidase (109) from, and reactivates it after, poisoning with arsenicals. *In vivo*, BAL protects against the vesicant action of lewisite and accelerates its elimination from the skin and its excretion in urine (110). A water-soluble derivative, BAL-glucoside, is suitable for intravenous injections and is able to counter the systemic effects of arsenical poisoning (111).

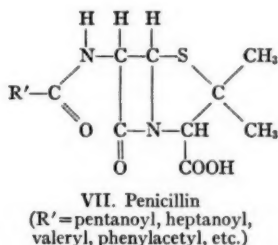
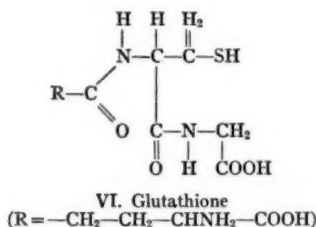
BAL and other dithiols are also useful, *in vitro* and *in vivo*, as antidotes against intoxication by compounds of antimony, mercury, cadmium, bismuth, gold, etc. (112 to 118).

BAL itself is not entirely harmless, partly because of its reducing properties, partly because of its affinity for heavy metals, and large doses inhibit several metal-containing enzymes (119, 120). BAL and other dithiols form sluggish oxidation-reduction systems

(E_0' for BAL = -0.150 v.), the reduced forms of which are not autoxidizable, but are easily oxidized in presence of catalytic amounts of heavy metals (121).

Thiol groups in enzymes.—The inhibition of thiol enzymes by ionizing radiations is largely due to oxidation of the thiol group and can be prevented or even reversed by glutathione (122). Singer (123) made the interesting observation that, whereas inhibition of D-amino acid oxidase by *p*-chloromercuribenzoic acid is independent of the substrate used, the inhibition of lipase depends on the molecular size of the substrate in a systematic way. This is interpreted to mean that in the case of lipase the inhibitor reacts with a group not directly involved in enzymatic activity, but nevertheless causes a change in enzyme configuration. The nephrotoxic action of tetrathionate is probably associated with the oxidation of essential thiol groups. Succinic dehydrogenase is inhibited *in vitro*, but not *in vivo* (124).

The discussion on thiol groups would not be complete without mentioning the remarkable structural resemblance between glutathione VI and penicillin VII to which Fischer (125) drew attention. Is glutathione an antagonist of penicillin? Inactivation of penicillin (and also of a number of other antibiotics) by cysteine and cysteine derivatives including glutathione has been described by Cavallito *et al.* (212) and by Cavallito (213). Glutathione was found to be less active than cysteine or cysteinylglycine. It is doubtful, however, whether this inactivation is due to competitive antagonism; the evidence is rather in favor of a chemical reaction between penicillin and thiol compound, since the effect depends on the length of the preliminary incubation of the inhibitor solution with penicillin and on the absolute as well as the relative concentrations of the components during this phase.



OXIDATION MECHANISMS AND PATHWAYS
THE TRICARBOXYLIC ACID CYCLE

Metabolism of acetate and acetoacetate.—The investigations of Weinhouse, Medes & Floyd (126) lend further support to the concept that acetoacetic acid is oxidized through the tricarboxylic acid cycle. Using C^{13} -acetoacetate, labeled in the carbonyl and carboxyl groups, they found that rat kidney homogenates produced increased amounts of citrate if acetoacetate, oxaloacetate and barium ions were present. The citrate was isolated and shown to contain an excess of C^{13} which was almost entirely confined to the two primary carboxyl groups. In similar experiments Buchanan *et al.* (127) have previously demonstrated the incorporation of C^{13} into ketoglutarate, succinate, and fumarate. Weinhouse *et al.* favor the view that condensation with oxaloacetate occurs after the breakdown of acetoacetate into acetyl groups. The primary condensation product is assumed to be an unsymmetrical derivative of citric acid formed by the addition of an acetyl compound, CH_3COX , to the carbonyl double bond of oxaloacetate. This would account for the observed distribution of C^{13} in ketoglutarate and at the same time obviate the relegation of citric acid into a side shunt of the tricarboxylic acid cycle. Though C^{13} -labeled acetate was oxidized by rat liver and kidney, citrate, added initially and recovered after the experiment, did not contain any excess C^{13} indicating that intermediates of acetate metabolism do not come into equilibrium with tricarboxylic acids. The authors conclude that acetoacetate is not an intermediate in the normal oxidation of acetate, in agreement with their previous conclusions (128). These results are supplemented by the experiments of Buchanan *et al.* (129) who show that acetate is not an intermediate in the oxidative metabolism of acetoacetate. They also find that α -ketoglutaric acid except in low concentrations has a striking effect in stimulating the rate of acetoacetate metabolism.

The conversion of acetate into acetoacetate in liver tissue has been studied by Crandall, Gurin & Wilson (130). With a washed tissue suspension in which the reaction occurred only in the presence of pyruvate carboxyl-labeled acetate yielded acetoacetate with the isotope equally distributed between carboxyl and carbonyl groups. From this the authors concluded that acetate either condensed with itself or condensed with a two-carbon fragment

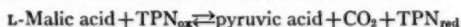
derived from pyruvate to form an equal mixture of carboxyl- and carbonyl-labeled acetoacetate.

Novelli & Lipmann (131) present evidence for the existence of a new coenzyme involved in the metabolism of acetate. The coenzyme (coenzyme A, coenzyme of acetylation) is a derivative of pantothenic acid; its concentration is decreased in pantothenate-deficient strains of *Proteus morganii*. Addition of pantothenic acid results in increased production of coenzyme A and at the same time in an increase of pyruvate oxidation. It is assumed that coenzyme A is required for the oxidation of acetic acid, rather than for the oxidation of pyruvic to acetic acid. Coenzyme A is a component of the enzyme system which catalyzes the acetylation of sulfanilamide in pigeon liver.

The utilization of acetate for the synthesis of fat by yeast has been demonstrated by White & Werkman (132).

Fluoroacetate seems to be a fairly specific inhibitor of acetate metabolism. It leads to accumulation of acetate in tissues oxidizing pyruvate (133) and inhibits the formation of citrate from acetate by yeast (134).

Carboxylation and decarboxylation.—Lwoff *et al.* (135, 136, 137) describe a direct oxidation of malate to pyruvate by a route not involving oxaloacetate in the mutant S of *Moraxella lwoffii*. They discuss the possibility of phosphorylated intermediates. Lichstein & Umbreit (138) also conclude that the oxidation of malate by *E. coli* produces a compound which is not oxaloacetate itself, since it is more stable and does not react with semicarbazide. This compound, like oxaloacetate, is converted to pyruvate by decarboxylation in an enzymatic process which requires the presence of biotin. In this connection the highly interesting, but also very puzzling results of Ochoa, Mehler & Kornberg (139) must be mentioned. These authors have purified an enzyme from pigeon liver which catalyzes the reversible reaction:



Since Utter & Wood (140) have shown that decarboxylation of oxaloacetate by liver extracts is reversible in presence of ATP, one might at first sight be inclined to explain the results of Ochoa *et al.* simply as an energetic coupling of pyruvate carboxylation with malate oxidation. However, quite apart from the different reaction mechanism which would entail reduction instead of oxidation of codehydrogenase II during the carboxylation reaction, the inac-

tivity of codehydrogenase I argues against the involvement of malic dehydrogenase. More recently Ochoa *et al.* (141) stated that the enzyme system also catalyzes the decarboxylation of oxaloacetate and that its activity is reduced in biotin deficiency. Clarification of the position occupied by β -keto acids in the scheme of carbon dioxide fixation will be eagerly awaited.

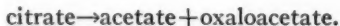
Meanwhile the first example of the reversibility of an α -decarboxylation has been reported by Wikén *et al.* (142). Lyophilized cells of *Staph. aureus* bring about a reversal of the dismutation of pyruvate into lactate and acetate + carbon dioxide in the presence of ATP. In cell-free extracts the reaction occurs in the absence of ATP, but is accelerated by its addition. The reversibility was demonstrated by incubating the enzyme preparation with pyruvate and isotopic bicarbonate. After the experiment the remaining pyruvate contained an excess of isotopic carbon in the carboxyl group.

Carboxylation of acetylmethylcarbinol to α -acetolactic acid by suspensions of *Staph. aureus* is suggested by Watt & Krampitz (143). Hastings *et al.* (144) found that 10 per cent of the carbon of glycogen formed by rabbit liver slices resulted from carbon dioxide fixation if pyruvate was the substrate, but only 2 per cent if glucose was the substrate.

Reversibility of the tricarboxylic acid cycle.—Lipton & Barron (145) succeeded in demonstrating the synthesis of acetylcholine by a soluble enzyme in presence of choline, ATP, an unknown coenzyme (Lipmann's coenzyme A?) and an "acetate-donor." The most efficient "acetate-donors" were citrate, *cis*-aconitate and acetoacetate. It is suggested that the effect of the first two acids is due to a reversal of the reaction



In support of this mechanism it is stated that the synthesis of acetylcholine in presence of citrate is accompanied by the production of a keto acid with the properties of oxaloacetic acid. The reaction



has previously been postulated by Deffner (146) on the basis of experiments with *Aerobacter*. Thus it would seem that all steps of the tricarboxylic acid cycle are reversible, with the probable exception of α -decarboxylations in animal tissues. The possible signifi-

cance of a tricarboxylic acid cycle in reverse for the mechanism of carbon dioxide assimilation in photosynthesis has repeatedly been pointed out.

OXIDATION OF FATTY ACIDS

The experiments of Weinhouse, Medes & Floyd (147) do not provide a rigid proof for the β -oxidation-condensation theory unless a secondary randomization of isotopic carbon between the carbonyl and carboxyl groups of acetoacetate can be excluded. This loophole has now been closed by Buchanan, Sakami & Gurin (148) working with acetoacetate labeled in either the carbonyl or the carboxyl position. Their results show that, at any rate in liver slices, a splitting into two-carbon fragments followed by random recondensation does not occur. Neither of these papers throws any light on the question whether cleavage of a two-carbon unit must precede oxidation at the next β -carbon or whether "multiple alternate oxidation" may spread along the fatty acid chain before fragmentation occurs. The latter mechanism is suggested by the fact, reported by Breusch & Ulusoy (149), that triacetic acid (β,δ -diketohexanoic acid) can be metabolized by liver tissue with the formation of more than one (usually about 1.3) mole of acetoacetate, showing that fragmentation into two-carbon residues and recondensation occur with a substance already oxidized in the δ -, as well as the β -, position.

Bartlett & Barron (133) have found that fluoroacetate (0.02 *M*) inhibits the formation of acetoacetate from higher fatty acids by liver slices while at the same time increasing the acetoacetate formation from β -hydroxybutyric or acetic acid. This indicates a specific affinity for the enzyme system responsible for β -oxidation. This is confirmed by the observations of Saunders (150) which show that of ω -substituted fluorocarboxylic acids only those with an even number of carbon atoms are toxic, whereas odd-numbered ones are not. Furthermore, the toxicity of compounds with an even number of carbon atoms disappears if the β -position is blocked by substitution.

NUCLEAR OXIDATION OF AROMATIC COMPOUNDS

Although the biological hydroxylation of the benzene ring has been known for a long time, the mechanism of the reaction was

obscure until the discovery by Boyland & Levi (151) of the conversion of anthracene into 1,2-dihydroanthracene-1,2-diol in rats and rabbits. These workers had already recorded the observation that the diols formed by the two species were not identical. Boyland & Shoppee (152) have now shown that both compounds are *cis*-enantiomorphs, but whereas the rat compound is optically active, the rabbit compound is racemic. Analogous results were obtained with naphthalene. Young (153) isolated 1,2-dihydronaphthalene-1,2-diol from the urine of rats after administration of naphthalene. The compound was laevorotary whereas a naphthalene diol isolated by Booth & Boyland (154) from rabbit urine was optically inactive. Weigert & Mottram (155) obtained spectroscopic evidence of the intermediate formation of 8,9-dihydro-3,4-benzpyrene-8,9-diol during the conversion of 3,4-benzpyrene to 3,4-benzpyrene-8-ol. It would appear, therefore, that a dihydroxylation of this type is a general feature of the biological oxidation of aromatic compounds. The diols are easily dehydrated to monophenols and it is probable that the phenolic metabolites of other aromatic hydrocarbons are decomposition products of primarily formed diols. This mechanism might be connected with another peculiarity frequently commented upon, viz., the fact that the phenolic hydroxyl group of the metabolite is usually at a position different from that which is most susceptible to oxidation *in vitro*.

Aromatic amines, whether acetylated or free, usually undergo nuclear oxidation *in vivo*. Recent examples are: the conversion of 2-acetylaminofluorene into 2-acetylamino-7-hydroxyfluorene (156), of sulfanilamide to 3-hydroxysulfanilamide (157) and of acetanilide to N-acetyl-*p*-aminophenol (158). If, on the other hand, the amine group is attached to an aliphatic side chain, the compound reacts with amine oxidase and is excreted without nuclear oxidation (159).

Little is known about the enzyme systems responsible for the nuclear oxidation of aromatic compounds. One such reaction, the oxidation of phenylalanine to tyrosine, can be regarded as a component of normal intermediary metabolism; it seems to be localized in the liver (160) and it is probably dependent on a specific enzyme system which is deranged in the metabolic error known as phenylpyruvic oligophrenia (161). On the other hand, carcinogenic hydrocarbons, such as 20-methylcholanthrene (162) or 3,4-benzpyrene (155), seem to be oxidized as soon as they permeate into the inte-

rior of almost any living cell with which they come into contact. Their oxidation is therefore more likely to be incidental to the normal metabolic activities of the cell, due perhaps to a peroxidatic mechanism, than to the action of a specific enzyme. But neither the continuous decomposition of hydrogen peroxide by blood catalase nor the action of soybean lipoxidase on linoleic acid is accompanied by a detectable oxidation, *in vitro*, of benzpyrene (163).

An interesting reaction, the aromatization of cyclohexane carboxylic acid to benzoic acid, has been studied by Dickens (164) using slices of rabbit tissues *in vitro*.

PHOSPHORYLATIVE ENERGY COUPLING

The question whether the oxidation of glyceraldehyde monophosphate to 1,3-diphosphoglyceric acid involves the formation (enzymatic or nonenzymatic) of a diphosphoglyceraldehyde is answered in the negative by Meyerhof & Oesper (165). Triose dehydrogenase catalyzes a thermodynamic equilibrium governed by the concentrations of glyceraldehyde phosphate, inorganic phosphate, codehydrogenase I and hydrogen ions. The mass action law for this equilibrium is so closely obeyed that the formation of an additional reaction partner in any considerable concentration can be excluded, "except in the presence of the oxidizing system and in its enzymic field of action."

Fatty livers of guinea pigs treated with carbon tetrachloride show an oxygen consumption and acetoacetate production considerably in excess of normal. Such livers also contain an increased concentration of adenosinediphosphate and phosphocreatine, indicating the formation of high-energy phosphate bonds at the expense of fat oxidation (166). Potter (167) further refined the technique of studying oxidative phosphorylation in tissue homogenates and discussed various factors influencing the results, especially the leak of phosphate bond energy resulting from phosphatase action. The problem of the maximum theoretical ratio of phosphorus to oxygen-uptake has been the subject of a stimulating discussion (168) suggesting that some previous figures are too high. Ochoa's values, e.g. (169), would be in better agreement with theory, if they were accepted at their face value instead of being corrected for a possible simultaneous action of adenosinetriphosphatase. However, as the authors frankly admit, some of their assumptions are subject to revision, as, e.g., the hypothesis

that only the oxidizing potential between cytochrome-*c* and substrate level is tapped for conversion into phosphorylation energy.

More and more reactions which were previously supposed to depend on the organization of the intact respiring cell are now shown to proceed in homogenates or even in cell-free extracts, provided ATP is supplied. Nothing could bring out more clearly the importance of the energy-rich phosphate bond as the general driving force behind endergonic biological reactions. Sometimes the effect of ATP merely consists in speeding up the reaction or it is observed only under anaerobic conditions. In these cases a limited endogenous supply of energy-rich phosphate bonds may be surmised. The following examples of ATP-controlled reactions may be mentioned (in addition to the carboxylation reactions previously referred to): the synthesis of urea (170, 171), hippuric acid (172), *p*-aminohippuric acid (173), and glutamine (175); the methylation of guanidoacetic acid (174) in liver homogenates; and the acetylation of sulfanilamide in liver extracts (131) and of choline in brain extracts (145).

Lardy & Elvehjem (176) tabulated a number of agents which are supposed selectively to inhibit the energy-coupling mechanism. It is characteristic for this type of inhibitor to accelerate exergonic processes, such as respiration and glycolysis, which are released from the braking effect of accumulated compounds with high-energy phosphate bonds. The action of azide, already included in Lardy & Elvehjem's list, has been further studied in this connection (177, 178). A selective inhibition of the energy-coupling mechanism might perhaps also account for the increased respiration of dystrophic muscle; it would at the same time provide an explanation for the rise of creatine excretion and the fall of creatinine excretion. Urinary creatinine is mainly derived from phosphocreatine according to Borsook & Dubnoff (179). If phosphocreatine synthesis were suppressed, creatinine excretion would decrease and the unphosphorylated creatine which the muscle is unable to retain would be excreted in greatly increased amount. Hummel (180) has indeed shown that this interpretation is probably correct. He found a depression of oxidative phosphorylations in muscle homogenates of dystrophic hamsters and guinea pigs which were not affected by addition, *in vitro*, of tocopheryl phosphate. The only flaw in the picture is the fact that oxidative phosphorylations were normal in muscle homogenates from dystrophic rabbits.

RESPIRATION AND GLYCOLYSIS OF
ANIMAL TISSUES

ACTIVATORS OF RESPIRATION

In connection with their studies on the activation of D-amino acid oxidase, reviewed in a preceding section, Edlbacher & Wiss (181, 182) examined the effect of amino acids on the oxidation of pyruvate by dilute extracts of rat liver and other tissues and again found histidine to be the most efficient activator. Large effects are produced by either the D- or the L-form in catalytically small amounts which do not noticeably decrease during the experiment. Maximum activation is regularly obtained if the rats are starved for twenty-four hours before the experiment. The oxidation of pyruvate by the activated extract does not go to completion (183). These results are interpreted by the authors as indicating the formation of active amino acid-enzyme complexes and it is implied that identical or similar complexes exist in the intact cell. It is suggested that the specific dynamic action of amino acids may be explained by this activation of respiration. Activation of enzymes by amino acids is of course no new observation and previous work has not been sufficiently considered by Edlbacher. References to the older literature are given in a paper by Lehmann & Pollak (184) who found a stimulation of phosphate transfer in muscle extract on addition of amino acids. Bailey (185) observed an activation of the adenosinetriphosphatase of a myosin preparation by amino acids and proposed that "the amino effect resides partly in the removal by co-ordination of heavy metals, partly in the increased heat stabilization of the enzyme and partly in some specific action of the dipole ion." Lehmann & Pollak (184) add a further suggestion; increase of the solubility of sparingly soluble salts. This would however require fairly high concentrations of amino acids, such as were actually used by these authors. Burk *et al.* (186) found that the inhibition of tissue respiration by carbon monoxide or by copper ions is specifically antagonized by histidine. From the facts quoted it would appear probable that the effects of amino acids are not specific for any particular group of enzymes and that their action is of a protective nature, directed against the hazards attendant upon progressive dilution and purification of enzymes *in vitro*.

The higher respiration of tissues in serum than in Ringer solu-

tion is due partly to the bicarbonate content and partly to a substance present in the ultrafiltrate, having the properties of an organic acid (187). The lowered respiration of liver slices in thyroidectomized rats is brought back to the normal level after injection of thyroxine (188). *In vitro*, thyroxine increases tissue respiration only after a long lag period during which it is converted into an active form; thyroglobulin on the other hand has an immediate effect (189).

BRAIN METABOLISM

The glycolysis of brain has been subjected to a thorough analysis by Meyerhof *et al.* (190, 191, 192). Their results throw into vivid relief the controlling part played by ATP and adenosinetriphosphatase and permit a rational explanation of many previously disconnected and puzzling observations. Their observations may be summarized thus: adenosinetriphosphatase is bound to structural elements and its activity is therefore high in homogenates and low in extracts. Consequently the concentration of ATP is high and steady in extracts, while low and rapidly declining in homogenates. Phosphate acceptors, such as hexoses or hexosemonophosphate, requiring a high concentration of ATP, are fermented slowly in the homogenate, but rapidly in the extract. The reverse is true for a phosphate donor, e.g., hexosediphosphate; its fermentation is slow in extracts, but rapid in a homogenate where the continuous dephosphorylation of ATP maintains at a high rate the discharge of high-energy phosphate from diphosphoglyceric and phosphopyruvic acids. The fermentation of hexosediphosphate in extracts can be accelerated by addition of purified adenosinetriphosphatase or by arsenate which breaks the coupling between dephosphorylation and the ATP-system.

With decreasing concentrations of ATP the affinity of brain hexokinase for fructose is lowered much more than for glucose. In homogenates where the ATP concentration is low the fermentation of glucose is therefore much more rapid than that of fructose, whereas in extracts the rate is about the same because of the high concentration of ATP. If ATP or other phosphate donors are added to a homogenate, or if the activity of adenosinetriphosphatase is lowered by inhibitors or by dilution, the difference between the fermentation rates of glucose and fructose disappears in the homogenate.

Adenosinetriphosphatase is very unstable in a water homogenate, in contrast to a Ringer homogenate. The properties of a water homogenate are therefore much closer to those of an extract than to those of a Ringer homogenate. The difference between water and Ringer homogenates is not due to cytolysis (there are hardly any intact cells even in a Ringer homogenate), but to the different activity of adenosinetriphosphatase. Owing to the opposing effects of the enzyme on the fermentation of glucose and of hexosediphosphate, addition of both substrates together produces a well-balanced fermentation system, whatever be the amount of enzyme present. Geiger's inhibitor of glycolysis which is bound to structural elements is, in addition to diphosphopyridine nucleotidase, mainly adenosinetriphosphatase since the inhibitory action is little affected if nucleotidase activity is checked by nicotinamide.

The optimum conditions for respiration, and aerobic and anaerobic glycolysis in water homogenates of brain have also been studied by Reiner (193). These preparations show no Pasteur effect.

A number of substances other than glucose, known to sustain the respiration of brain slices *in vitro*, such as fructose, glutamate, lactate, pyruvate and succinate, are nonetheless unable to maintain the electrical activity of brain or to relieve symptoms of hypoglycemia in eviscerated animals. Klein *et al.* (194, 195) determined the concentration of these substances in cat brain after intravenous injection and found that the rate of transfer from blood to brain was insufficient to provide an effective concentration in brain. That the difference between glucose and other substrates may be only relative is suggested by the observations of Mayer-Gross & Walker (196) who could relieve hypoglycemic coma in man by intravenous injection of L-glutamic acid. Consciousness was restored while the blood sugar level was well below that which arrests the coma if glucose be injected. On the other hand, the effect of glutamic acid seems to be neither as prompt nor as sure as that of glucose.

OTHER TISSUES

Gastrointestinal tract.—The respiration and glycolysis of gastric tissues has been studied by Lutwak-Mann & Barrett (197) and by Davenport (198).

A controversy on the mechanism of hydrochloric acid production in gastric mucosa has developed between Conway & Brady (199) and Davies, Longmuir & Crane (200). There seems to be substantial agreement on the following points: (a) the secretion of hydrogen ions liberates an equivalent amount of alkali within the cell which is neutralized by carbonic acid formed from the carbon dioxide of blood or tissue fluids, a process catalyzed by the gastric carbonic anhydrase [Davies *et al.* (200)]; (b) the secretion of hydrogen ions probably involves an ion exchange across the cellular membrane; potassium ions move in while hydrogen ions move out. The excess potassium ions then leave the cell again, paired with chloride ions. The chloride stores of the cell are replenished by exchange of bicarbonate (from the cell) and chloride (from the blood) (Conway & Brady). There is however disagreement on how hydrogen ions are generated. Conway & Brady suggest that they are produced by the dehydrogenation of triose-phosphate to phosphoglyceric acid or some similar reaction in a cyclical process. Dehydrogenation which produces acid is followed by hydrogenation which produces an equivalent amount of alkali. The acid is secreted into the gastric lumen whereas the alkali is carried away by the circulation. This simple idea is criticized by Davies *et al.* on grounds which do not seem convincing.

Rosenthal (201) measured the metabolism of the duodenal mucosa of the rabbit. In contrast to the findings of Dickens & Weil-Malherbe (202) with intestinal mucosa of rats and mice he found a very small aerobic glycolysis and a strong Pasteur effect ($Q_G^{O_2}=0.1$; $Q_G^{N_2}=7.7$). He concludes that the exceptional behavior of murine mucosa is a species peculiarity, if not an artefact. The latter possibility can be excluded, since Dickens & Weil-Malherbe have shown that the high aerobic glycolysis of the mucosa may be observed in the intact intestinal wall and cannot, therefore, be attributed to the fragility of the detached membrane.

Cardiac & skeletal muscle.—A stimulation of the respiration of isolated cardiac muscle by digitalis glucosides has been observed independently by Wollenberger (203) and Libert (204, 205). According to Wollenberger 2 to 3×10^{-7} M ouabain increases the respiration of guinea pig heart by about 50 per cent in the presence of glucose or lactate. Only brain cortex responds similarly, but is one-fifth less sensitive than heart. Digitoxin is two to three times, k-strophanthin one-half as potent as ouabain. Libert, too, found

that digitaline and scillarene selectively increased the respiration of rat myocardium in presence of glucose or lactate.

Gibson & Long (206) have studied pyruvate oxidation by a dialyzed preparation of ox heart which requires the presence of inorganic phosphate, ATP, fumarate and Mg^{++} for complete oxidation.

An impairment of muscle respiration in choline-deficient rats is reported by Abdon & Borglin (207). Choline has no effect *in vitro*, but a complex choline compound which is considered to be a precursor of acetylcholine normalizes the defect. It is without effect on normal muscles.

Semen.—Fructose is the only fermentable reducing sugar present in semen. Under anaerobic conditions fructolysis is the only source of energy for spermatozoa, but aerobically mannose and glucose can also be utilized (208). Human spermatozoa are unique in having a low respiration and a very high aerobic and anaerobic glycolysis. They are also very sensitive to high oxygen tensions and to thiol inhibitors (209).

Tumors.—*p*-Phenylenediamine causes a greater stimulation of respiration in slices of three transplantable mouse melanomas than in malignant tumors generally (210). This may be connected with a higher level of oxidation-reduction potential in these tumors, leading to a high ratio of oxidized:reduced cytochrome-*c*. There is no difference in this respect between melanotic and amelanotic melanomas.

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² References which have been drawn from earlier volumes of the *Annual Review of Biochemistry* are as follows: (3, 7, 30, 39, 57, 86, 128, 211) **16**; (53, 62, 63, 101, 127, 140) **15**; (147, 160, 169, 176) **14**; (13) **13**; (77, 83, 184) **12**; (146) **10**; (21) **9**; (20) **8**; (16) **7**; (151) **6**.

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NONOXIDATIVE ENZYMES

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This review covers selected research papers appearing between December 1, 1946 and December 1, 1947. A few papers published in European journals from 1940 to 1946 have been included, since it is assumed that most of these have not been seen by previous reviewers.

Acetylation.—The coenzyme for acetylation is a pantothenic acid derivative, and occurs in liver and in brain, according to Lipmann *et al.* (1).

Novelli & Lipmann (2) find that the microorganisms *L. arabinosus* and *Proteus morganii* convert pantothenic acid into coenzyme A. Coenzyme A is claimed to be a factor required for the reaction in the liver whereby acetate reacts with ATP to form a compound with the properties of acetyl phosphate.

Acetylcholine - phosphocreatinase. — Nachmansohn (3) has claimed that an enzyme in nerve causes acetylcholine and phosphocreatine to react, thus producing the energy required for the nerve impulse. Rapp (4) now shows that this enzyme competes with choline esterase for the acetylcholine (liberated) in nerve. He prepared the enzyme from frog's sciatic nerves, using a blender. When added to either acetylcholine or phosphocreatine there was no action, but when both acetylcholine and phosphocreatine were present both were decomposed. The reaction appears to be mole for mole. Procaine inhibits the enzyme competitively.

Adaptive enzymes.—Spiegelman, Reiner & Morgan (5) conclude that the adaptation of yeast to galactose fermentation consists in a change of the apoenzyme. Both the polygalacturonase and pectinesterase of *Penicillium chrysogenum* are adaptive enzymes according to Phaff (6), although not in a strict sense. Gäumann & Böhni (7) have found that in *Botrytis cinerea* Pers. the pectinase is a constitutive enzyme, while the unspecific pectase is very largely an adaptive enzyme.

Adenosine deaminase.—This enzyme has been prepared from calf intestinal mucosa by Zittle (8) and found to deaminate adenosine and dextroxyriboadenosine, but not adenine. The deaminase

has a broad pH optimum at 7.0 and is active at pH 5.9, where phosphoesterases are inactive. The activity of the enzyme was determined directly through the formation of ammonia, or indirectly through the absorption of carbon dioxide into the system made alkaline by liberation of the ammonia. Zittle suggests that adenosine deaminase can be employed for the estimation of adenosine.

Adenosinetriphosphatase.—Mommaerts (9) finds that the liberation of inorganic phosphate from ATP in dog muscle occurs at a rate of 0.003 mg. of phosphorus per min. per mg. of myosin. From Lundsgaard's (10) data, the rate of liberation in frog muscle is at least 0.2 mg. of phosphorus per minute during contraction. Hence, Mommaerts concludes, this hydrolysis of ATP cannot be the source of energy for muscular contraction. In a later article Mommaerts & Seraidarian (11) have reported upon the adenosinetriphosphatase activity of myosin and actomyosin. There is no enzyme activity in the absence of salts. While potassium chloride and calcium chloride activate, magnesium chloride inhibits in the presence of other salts. The pH effect is complex. In stabilizing buffers or at low temperatures there are two pH optima if calcium ions are present; these are at pH 6.2 to 6.5 and at pH 9.2. The greatest adenosinetriphosphatase activity was found at pH 9.2 in the presence of calcium ions and in glycine buffer. The authors conclude that myosin-adenosinetriphosphatase cannot be responsible for the main liberation of phosphate in contracting muscle and, therefore, cannot have the role which has been ascribed to it.

Polis & Meyerhof (12) calculate that if all the adenosinetriphosphatase is myosin then its turnover number is only 3×10^2 , whereas other phosphatases have been found to have turnover numbers of 1×10^4 to 1×10^6 at 38°C . These authors have adsorbed adenosinetriphosphatase on a basic lanthanum salt at pH 8.0 and have then eluted it with a solution containing potassium chloride, potassium cyanide, and ATP. This achieved a threefold increase in activity. In low concentrations, sulfhydryl inhibitors, such as iodoacetamide, iodoacetate, and iodosobenzoate activate adenosinetriphosphatase 30 to 70 per cent. Glutathione counteracts both the activating and inhibiting effects.

The adenosinetriphosphatase of human serum is found by Meister (13) to have one pH optimum at 4.5 to 5.0 and another at pH 8.5 to 9.0. Perhaps two enzymes are present.

Banga, Guba & Szent-Györgyi (14) look on myosin, the chief protein of muscle, as a system of substances. The enzymes present are called "Protins." These are:

(a) ATP-P-protin = adenosinetriphosphatase; (b) ATP-C-protin = substance concerned with contraction of myosin; (c) ATP-N-protin = deaminase of adenosinetriphosphate; (d) ADP-P-protin = enzyme causing dephosphorylation of adenosinediphosphate; (e) ADP-C-protin = substance concerned with myosin contraction in the presence of adenosinediphosphate; and (f) ADP-N-protin = deaminase of adenosinediphosphate.

AMYLASES

Crystalline pancreatic amylase.—One of the outstanding achievements of the year is the crystallization of amylase from pig pancreas by Meyer, Fischer & Bernfeld (15). Here, the greatest obstacle was the great instability of the partly purified amylase. The enzyme was stabilized by boiled amylase or an amylase dialysate. The crystallized amylase, however, was found to be very stable at pH 6.9 at 2°C. The final product was threefold more active than the purest product obtained by Sherman and twice as active as that of Willstätter & Waldschmidt-Leitz. It will be recalled that Sherman and his collaborators (16) claimed to have crystallized pancreatic amylase, but did not publish additional articles describing their method of purification. Meyer *et al.* (15) give data for the light absorption, solubility and electrophoretic mobility of their amylase.

Engel (17) has found that in resting grains of wheat, rye, and barley the bran and aleurone cells contain no amylase. The sub-aleurone layer which contains starch is rich in amylase. The inner endosperm contains moderate quantities of amylase and the part lying close to the germ is very rich in the enzyme.

The action of malt amylases on hexasaccharides with maltose linkages (dextrin) has been investigated by Myrbäck (18). Such a dextrin is completely split by β -amylase. α -Amylase transforms the dextrin wholly into fermentable sugar, consisting of maltotriose, maltose, and glucose. The α -amylase did not attack the maltotriose.

Sandstedt & Beckord (19) have investigated the amylolytic enzymes and the amylase inhibitor of the sprouting wheat kernel. The pericarp contains an α -amylase capable of digesting starch

granules, but the endosperm contains none, or only traces. An amylase inhibitor which inhibits salivary amylase, although not the natural wheat or malt amylases, appears in the endosperm at approximately the time that the embryo reaches full length. Andersch (20) has described a method for the determination of serum amylase, using β -amylase as the substrate.

Arginase.—Thompson (21) has prepared arginase of high purity, using the method of Mohamed & Greenberg (22). The product was a green-brown solution and showed three or four constituents in the Tiselius electrophoresis apparatus. Catalase was present. By the addition of cobaltous or manganous salts at pH 4 and prompt adjustment to pH 9 with phosphate buffer colored impurities were precipitated. A colorless arginase with the properties of an albumin remained in solution.

An enzyme in the intestinal epithelium which attacks D-arginine but not L-arginine to form urea and ornithine has been reported by Kotake & Mabuti (23). The enzyme is somewhat activated by manganese and is inhibited by L-ornithine.

Blood coagulation.—Ware, Guest & Seegers (24) describe a plasma accelerator factor (25) which aids in the activation of prothrombin to thrombin when thromboplastin and calcium ions are present. If this factor is present the amount of thrombin formed is greater than in its absence.

Staphylocoagulase, an enzyme from pathogenic streptococci, has been reported upon by Agnew, Kaplan & Spink (26). It forms a fibrin clot when the organisms are added to citrated human plasma. This action is inhibited more readily by streptomycin than by penicillin.

Blood group enzyme.—The blood group enzyme of Schiff (27) has been investigated by Neuda (28) who believes it to be identical, possibly, with a substance which causes the erythrocytes of negroes, suffering from sickle cell disease, to assume a sickle shape. The early workers obtained the blood group enzyme from feces, or saliva. It was found to destroy the antigens of pepsin, trypsin and peptone, also of blood groups A, B, and O. Neuda now finds that the enzyme can be obtained from plasma, or serum, and that it acts not only on negro erythrocytes, but also on erythrocytes of diseased Caucasians. In some instances the enzyme could act directly and did not need to be "enriched" by a twenty-four-hour incubation of sterile plasma with sterile nutrient broth.

Carbonic anhydrase.—Bradfield (29) has found carbonic anhydrase to be present in the leaves of many plants in low concentration. Unlike animal carbonic anhydrase it is not inhibited by sulfanilamide, but is inhibited by cyanide and by azide. The enzyme is not present in the chloroplasts. Day & Franklin (30) report the presence of carbonic anhydrase in *Sambucus Canadensis*. It is absent or of irregular occurrence in other plants.

Carboxypeptidase.—Fruton & Bergmann (31) found that phenylpyruvylamino acids are hydrolyzed by crystalline carboxypeptidase. Phenylpyruvyl-L-phenylalanine is split most rapidly, phenylpyruvyl-L-glutamic acid very slowly, and phenylpyruvylglycine the most slowly of all three.

Crystalline carboxypeptidase has been investigated by Putnam & Neurath (32). The molecule has a low degree of asymmetry and the molecular weight of the eight-times crystallized enzyme is 32,000. The isoelectric point is at pH 6.0. The authors have studied the kinetics of the hydrolysis by carboxypeptidase of chloroacetyl-L-tyrosine and of chloroacetyl-L-phenylalanine. Formaldehyde has an inhibitory effect as already reported by Hoffmann & Bergmann (33).

Neurath, Elkins & Kaufman (34) have found that carboxypeptidase does not attack D-carbobenzoxyglycylphenylalanine and the presence of this D isomer does not inhibit the action of carboxypeptidase upon the L isomer.

Catalases.—Herbert & Pinsent (35) have obtained catalase from *Micrococcus lysodeikticus* in crystalline form after lysing the cells by use of lysozyme. Assuming four atoms of iron to be present, the molecular weight is 226,000 to 248,000. The "*Kat. f.*" is declared to be 90,000. No iron-biliverdin groups are present.

The protein components of crystalline horse liver catalase and crystalline horse erythrocyte catalase have been studied by Bonnichsen (36). The nitrogen distribution and amino acid content were the same in both. The absorption in the ultraviolet was also the same. The sera of rabbits immunized with horse liver catalase precipitated both horse liver catalase and horse blood catalase, while human liver catalase gave no precipitate. Bonnichsen found crystalline human erythrocyte catalase to have a "*Kat. f.*" of 50,000, while crystalline horse erythrocyte catalase had a "*Kat. f.*" of 65,000. The value of 100,000 previously reported by Agner for horse erythrocyte catalase is probably much too high (37).

Chance (38) has discovered that catalase forms an intermediate compound with hydrogen peroxide. This compound reacts with acceptors and inhibitors. Its absorption spectrum between 380 and 430 $m\mu$ is shifted, as compared with that of catalase, slightly towards the red. The reviewer considers this work to support the hypothesis of Sumner & Dounce (39) that catalase reacts with hydrogen peroxide to form catalase peroxide and water and that this catalase peroxide next reacts with a second molecule of hydrogen peroxide to form regenerated catalase, water, and molecular oxygen.

Cholinesterase.—Augustinsson (40) has shown that during cataphoresis there is a large difference between the pseudocholinesterase of horse serum and the specific cholinesterase of horse erythrocytes. The specific cholinesterase is more sensitive to pH than is serum cholinesterase. Augustinsson agrees with earlier workers that the true cholinesterase of erythrocytes is identical with the cholinesterase of the brain. He believes that many cholinesterases exist. In *Helix pomatia* the cholinesterase of the blood is different from that of the dart sac (41). Both enzymes are inhibited by physostigmine.

Klein (42) has found that thionine, toluene blue and methylene blue inhibit cholinesterase, but the leuco forms have less effect than the oxidized forms. The inhibiting action is independent of the oxidation-reduction potential.

The antimony electrode and an ordinary potentiometer have been employed by Delaunois & Cosier (43) for the determination of cholinesterase. The value for the spontaneous hydrolysis is subtracted.

Riker & Wescoe (44) find that after the cholinesterase of cat skeletal muscle is inactivated by diisopropylfluorophosphate the muscle still contracts in a characteristic manner when the arterial blood supplying the muscle contains acetylcholine. Prostigmine also causes the muscle to respond in the absence of cholinesterase. It is concluded that prostigmine should be classified pharmacologically with the choline esters. The authors doubt that cholinesterase plays a primary role in the mechanism of acetylcholine action.

Frog nerve poisoned with diisopropylfluorophosphate still conducts impulses with normal action potentials but the cholinesterase is entirely inactivated according to Boyarsky, Tobias & Gesard

(45). Hence, they conclude that cholinesterase is not essential to nervous conduction.

Mendel & Hawkins (46) find that at low concentrations diisopropylfluorophosphate inhibits pseudocholinesterase selectively when both true and pseudo-esterases are present. The studies of Nachmansohn, Rothenberg & Feld (47) show that the inhibition of cholinesterase by diisopropylfluorophosphate is reversible for a certain length of time, depending upon temperature and the concentration of inhibitors. The inactivation, *in vitro*, requires much less diisopropylfluorophosphate than inactivation *in vivo*. This difference is possibly due to a lipid membrane surrounding the axon and acting as a barrier. The authors note that the *nucleus caudatus* of the ox is an excellent source of cholinesterase.

Paléus (48) states that the specific cholinesterase of erythrocytes is bound to the erythrocyte membrane and cannot be eluted. Barnard (49) finds his cholinesterase preparations to contain iron.

A manometric method for cholinesterase determination is described by DuBois & Mangun (50) who state that the enzyme is inactivated by hexaethyl tetraphosphate.

Mentha, Spring & Barnard (51) describe a method for the extraction of cholinesterase from human erythrocytes: a chilled paste of erythrocytes is extracted for three hours with 0.1 volume of 0.85 per cent sodium chloride containing enough ammonia to give a pH of 8.3; the supernatant fluid contains the enzyme; all ammonia present must be removed at once. Thus prepared, the enzyme will keep indefinitely in the ice chest.

Augustinsson (52) has made a survey of the occurrence of cholinesterase in invertebrates. He finds that the use of acetyl- β -methylcholine and benzoylcholine, as a means of distinguishing between different cholinesterases, is not in itself sufficient. Certain cholinesterases, while able to split acetylcholine, were unable to split either of the two esters mentioned above.

Choline acetylase.—Minz (53) finds that anaerobic acetylcholine formation by leech muscle is increased by addition of cocarboxylase. The cocarboxylase is believed to cause a formation of acetic acid through decarboxylation of pyruvic acid. Previously choline acetylase had not been found in muscle or liver extracts but Nachmansohn, Berman & Weiss (54) have prepared extracts of acetone-pigeon breast muscle and of guinea pig skeletal muscle which contain the enzyme. A solution of muscle to which have been added

choline acetate, eserine, potassium ions, magnesium ions, ATP, and the coenzyme will form acetylcholine. No choline acetylase was found in either kidney or liver.

Lipton & Barron (55) have demonstrated a synthesis of acetylcholine, using a water extract of acetone-brain. It was necessary to have present potassium ions, choline, a coenzyme, ATP and also citrate, *cis*-aconitate, or acetoacetate. These substances probably yield "active" acetate, which acetylates the choline. The coenzyme can be obtained either from boiled yeast or from animal tissues.

Cozymase nucleosidase.—Spaulding & Graham (58) have investigated the cozymase nucleosidase of rat tissues. It splits nicotinic acid amide from cozymase (diphosphopyridine nucleotide) and is most active at pH 7.2. The enzyme is inhibited by α -tocopheryl phosphate, as was postulated by Govier *et al.* (59).

Codecarboxylase.—Karrer & Viscontini (56) have phosphorylated pyridoxalacetal in pyridine, using phosphorus oxychloride. The purified crystalline product, containing phosphate on the phenolic hydroxyl, was found to function as a codecarboxylase even better than a mixture of pyridoxal and ATP. This is contested by Gunsalus & Umbreit (57).

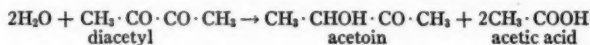
Cysteine desulfurase.—Fromageot & Grand (60) have concluded that since cysteine desulfurase is inactivated by 10^{-3} *M* HCN and by even lower concentrations of hydrazine, phenylhydrazine, hydroxylamine and semicarbazide, a carbonyl group, necessary for enzyme activity, must be present in the enzyme molecule. No heavy metal is believed to be present. The enzyme contains two other active groups, $-\text{COO}^-$ and $-\text{N}^+$, according to Fromageot & Grand.

Dehydropeptidase.—According to Yudkin & Fruton (61) swine kidney dehydropeptidase, upon dialysis, loses its activity towards glycyldehydrophenylalanine. This loss in activity is fully restored if traces of zinc chloride are added.

Dextran-synthesizing enzyme.—Hehre (62) has studied the reaction which occurs when the dextran-synthesizing enzyme from *Leuconostoc mesenteroides* acts upon sucrose to form dextran and fructose. The synthesis of dextran followed a first order reaction in systems containing 1 per cent of sucrose, but resembled a zero order reaction in systems containing 5 per cent of sucrose. The addition of purified dextran to enzyme-sucrose mixtures did not

increase the initial velocity of the reaction. However, small amounts of dextran were present in the enzyme used; hence this does not exclude dextran as an activator. The dextran-synthesizing enzyme is not inhibited by azide, cyanide, fluoride, or iodoacetate.

Diacetyl mutase.—Green, Stumpf & Zarudnaya (63) have discovered an enzyme, diacetyl mutase, which catalyzes the reaction:



The enzyme occurs in the muscles of various animal species and in high concentration in pigeon breast muscle.

Enzyme action at a distance.—Rothen (64) found that a film of antigen deposited on a metal slide was capable of reacting with an antibody even in spite of being covered with a layer of barium stearate, octadecyl amine, or formvar (polyvinyl formaldehyde). Reaction occurred even when the film of material was 200 Å thick. It was shown that trypsin could act on such a protein antigen as bovine albumin even when the film was present in a thickness of something more than 100 Å. Rothen does not give convincing proof that the films through which trypsin acted did not contain holes. If this hypothesis of enzyme action at a distance were valid we could expect no enzyme-substrate complexes to exist and, furthermore, we could more readily understand why it is possible for catalase to possess a turnover number of five million.

Enzyme inhibitors.—Bowman (65) has classified the antitryptic factors of the soybean as follows: (a) the factor that can be precipitated by alcohol (66) and found by Kunitz (67) to be a crystallizable globulin; (b) a factor which is soluble in alcohol, but insoluble in acetone. According to Ham & Sandstedt (68) the latter does not precipitate upon heating with 25 per cent trichloroacetic acid, nor is it salted out by ammonium sulfate of 40 per cent concentration. Borchers, Ackerson & Sandstedt (69) have now carried out further investigations of inhibitor (b). It is extracted from soybean meal with dilute hydrochloric acid. It does not compete with trypsin for the substrate. The reaction involved is an equilibrium reaction. Incubating the inhibitor with trypsin for twenty-four hours does not destroy the inhibitor. Borchers *et al.* (69) define a unit of the inhibitor as that amount which will reduce 1 trypsin unit to 0.75 units.

Trypsin inhibitors have been found in many of the legumes by

Borchers & Ackerson (70) but not in all. None were found in jack beans, lentils, garden peas, or horse beans. Inhibitors were not present in the seeds of nonlegumes. Kunitz (71) finds that his crystallizable soybean trypsin inhibitor is a stable protein of the globulin type; the molecular weight is about 24,000 and the isoelectric point is at pH 4.5. It unites with an equal weight of trypsin to form a stable compound. The inhibitor inactivates chymotrypsin only slightly and does not inactivate pepsin. If first denatured, it is readily digested by pepsin and less readily by chymotrypsin and trypsin.

Blaschko, Chou & Wajda (72) find that the cholinesterase of the central nervous system is little inhibited by paludrine, although some of the other esterases are inhibited.

Lewisite inactivates all sulfhydryl enzymes except D-amino acid oxidase [Barron *et al.* (73)] and BAL 2,3-dimercaptopropanol) reactivates these enzymes.

Webb & Van Heyningen (74) have observed that BAL inhibits metal-containing enzymes, with the exception of the cytochrome system.

Esterases.—The distribution of esterase in wheat, rye, and barley grains has been investigated by Engel (75) and his colleagues. The aleurone cells, as well as the germ part of wheat and rye grains, were rich in esterase. There was a negligible quantity in the endosperm of all cereals tested. With barley there was considerable autolysis and esterase determination was impossible. The aleurone cells of wheat, rye and barley are also rich in proteinase and dipeptidase (76); the endosperm contains negligible amounts of these two enzymes and the germ only moderate amounts of proteinase, but much dipeptidase.

Lagerlöf (77) has differentiated serum esterase from pancreatic lipase, using a stalagmometric method. The esterase is readily inactivated by atoxyl and is not activated by calcium oleate. The lipase is resistant to atoxyl and is greatly activated by calcium oleate.

Fibrinolysin. Dyckerhoff & Jacober (78) have observed that fibrinolysin (fibrinolase) has proteolytic activity and optimum activity at pH 7. They state that its activity as a thrombin is slight. It inhibits the first phase of blood coagulation and it is unlikely that this inhibition is due to a proteolytic destruction of the prothrombin.

The principles which affect the hydrolysis of fibrin and fibrinogen have been classified by Loomis, George & Ryder (79) as follows: *profibrinolysin*, the inactive form of fibrinolysin, convertible to fibrinolysin by streptokinase, organic solvents and other enzyme activators; *fibrinolysin*, the activated lytic principle from serum or plasma, which dissolves fibrin and changes fibrinogen so that it will not clot with thrombin, and also destroys prothrombin; *streptokinase*, an exotoxin of certain strains of hemolytic streptococci; *antifibrinolysin*, a fibrinolysin inhibitor in plasma; *antiprofibrinolysin*, an antifibrinolysin inhibitor; and *antistreptokinase*, a substance in plasma which inhibits streptokinase. A paper by Astrup & Permin (80) is similar to that of Loomis, George & Ryder. The presence in animal tissues of a fibrinokinase is also reported.

Seegers (81) finds that fibrinolysin destroys prothrombin and that it does not convert prothrombin into thrombin. The natural protease (fibrinolysin) of several plasma protein fractions resembles pancreatic trypsin in its fibrinogenolytic, fibrinolytic and thromboplastic effects. These actions are inhibited by crystalline trypsin inhibitors from the pancreas and from the soybean. This protease is believed to participate in the blood clotting system [Ferguson, Travis & Geheim (82)]. Glazko (83) claims that thrombin is not inactivated by fibrinolysin.

Two tests for fibrinolysin, using fibrin formed by the action of thrombin on fibrinogen, have been described by Permin (84). He has found that the fibrinokinase of pig heart is insoluble in water, phosphate solution and other solutions. Most fibrinokinase occurred in erythrocyte stroma or in tissues of calf embryo, pig or rabbit. Tissues of the hen contained none. When tissue containing fibrinokinase was added to cow plasma there was no formation of fibrinolysin, owing to the presence in the plasma of antifibrinolysin. The profibrinolysin could be obtained free from antifibrinolysin by 0.33 saturation of plasma with ammonium sulfate. Bovine profibrinolysin could not be activated by streptokinase. Human profibrinolysin was activated by tissue fibrinokinase, but not to the same extent as by streptokinase. Bovine fibrinolysin was precipitated completely by dialysis.

β -Glucuronidase.—A method for the estimation of β -glucuronidase activity is described by Talalay, Fishman & Huggins (85), using phenolphthalein mono- β -glucuronide as substrate. The phenolphthalein which is liberated is determined colorimetrically.

The pH optimum of glucuronidase is found to be at pH 4.5 at 38°C. in 0.1 *N* acetate buffer. Fishman (86) finds that both natural and synthetic estrogens cause an increase in the concentration of β -glucuronidase of the mouse uterus. He considers that glucuronide formation is a "metabolic conjugation" rather than a detoxication. β -Glucuronidase has been prepared from liver, kidney and spleen by grinding in a Waring blender, bringing to pH 5.0, leaving at 38°C. for one-half hour, centrifuging down the protein impurity and then salting out the β -glucuronidase with an equal volume of saturated ammonium sulfate [Fishman & Talalay (88)].

Tissues from malignant neoplasms of various organs have been found by Fishman & Anltan (89) to contain from 100 to 3600 per cent more β -glucuronidase than uninvolved adjacent tissue. This high level is thought to indicate a metabolic response to high concentrations of estrogen or some closely related substance. Kerr & Levvy (90) have found an increase in the β -glucuronidase content of tissues after injecting toxic compounds (menthol, carbon tetrachloride, chloroform) which caused tissue damage. They believe that the increase in β -glucuronidase is associated with tissue regeneration.

Histidase and urocaninase.—Edlbacher & Viollier (91) have found that it is possible to separate histidase from urocaninase. Both of these enzymes occur in liver. Histidase was inhibited by all imidazole compounds that were tested. Urocaninase, which acts on urocanic acid, was inhibited by neither L- nor D-histidine. Histidine, it is claimed, can be broken down in two ways: (a) Histidase opens the imidazole ring; (b) histidine is deaminized with the formation of urocanic acid (β ,4-imidazole acrylic acid) which is next hydrolyzed by urocanase to L-glutamic acid [Edlbacher & Heitz (92)].

Histochemistry.—Ohlsen (93) made a histochemical study dealing with the distribution of certain enzymes in the gastric mucosa of the pig. Dipeptidase occurred in considerable amount in the pit epithelium, while aminopolypeptidase was in considerable concentration in the chief cell region of the fundus. A characteristic feature was the dormancy of the dipeptidase in the surface regions of the mucosa. The amount of pepsin in the pylorus was slight. Carboxypeptidase was absent and urease was absent or present in doubtful amounts.

Kugler & Bennett (94) employed the histochemical method of

Gomori (95) to examine sections of germinating maize kernels, obtained by the freezing technique, for acid phosphatase. The enzyme was found present in the embryonic axis, scutellum and aleurone layer.

A histochemical method for the detection of phosphorylase in soybeans has been described by Yin & Sun (96). The beans are soaked in water twelve hours and then cut into thin sections. These sections, upon incubating in 1 per cent Cori ester in buffer saturated with toluene, form starch. Other plant tissues which may contain preformed starch can be used if the starch is first removed by keeping in darkness for a certain length of time. In the soybean, phosphorylase was especially concentrated in the root cap. A less intense reaction occurred in the root tip and lateral buds. Still less phosphorylase was found in the young leaves, stem tip, and hypocotyl.

Hyaluronidase.—Monroy (97) finds that the hyaluronidase of sea urchin sperm dissolves the jelly coat of sea urchin eggs, while hyaluronidase from bull testis does not. Hechter & Scully (98) report that normal serum inhibits hyaluronidase *in vitro*, but not *in vivo*.

The viscosimetric assay of hyaluronidase has been critically studied by Swyer & Emmons (99). They find that within certain limits the fall in flow time of buffered hyaluronate-hyaluronidase solutions is proportional to the logarithm of the concentration of the enzyme. The error is less than 10 per cent. Leonard & Perlman (100) have adapted the turbidity method of Kass & Seastone (101) for determination of the hyaluronidase activity of semen and tissue. Enzyme activity is expressed in turbidity-reducing units. According to Leonard, Perlman & Kurzrok (102) if rat testis homogenate be incubated with rat seminal vesicle tissue, the quantity of hyaluronidase present is increased. Swyer (103) finds that in the semen of men, rabbits, bulls and boars there is a close correlation between the hyaluronidase content and the sperm density. In the case of dogs and fowls such correlation is lacking. Hyaluronidase is found to be liberated from rabbit sperm by freezing, or by keeping the sperm in water at 0°C. for twenty four hours [Swyer (104)].

Lecithinase.—Macfarlane & Knight (105) found the toxin of *Cl. welchii* to contain lecithinase which hydrolyzes lecithin to a diglyceride and phosphoryl choline. A manometric method for measuring the activity of this lecithinase, based upon the liberation

of carbon dioxide from bicarbonate buffer through the production of acid (phosphoryl choline), has been devised by Zamecnik, Brewster & Lipmann (106). Lecithin interferes with the combining of lecithinase (alpha toxin of *Cl. welchii*) with its antienzyme or antitoxin (107). However, if the enzyme and antitoxin are first allowed to unite lecithin exerts no effect.

Režek (108) reports that the hepatopancreatic juice of the snail splits lecithin into fatty acids, glycerol, phosphate, and choline. Enzyme action occurs between pH 1 and pH 10, but most rapidly at pH 6 to 7. One of the intermediate products is glycerophosphorylcholine. Fatty acids are formed at the start of the reaction and practically all of the hydrolyses begin at the same time.

Lipase.—Archibald (109) has devised a method for the determination of lipase activity wherein a completely water-soluble substrate is employed ("Tween 20," a polyoxyalkalene derivative of sorbitan monolaurate).

Krukovsky (110) finds that the photoinactivation of milk lipase is not caused by a production of hydrogen peroxide, formed through oxidation of the ascorbic acid.

The optimum pH for the action of human gastric lipase upon tripropionin, tributyrin and tricaproin has been shown by Schønheyder & Volquartz (111) to lie between 5.5 and 5.8, while tricaprin, trilaurin, and tristearin are hydrolyzed most rapidly at pH 7.2, 7.3 and 7.9 respectively. Addition of calcium chloride causes the pH optimum for hydrolysis of trilaurin to shift towards the acid side by 1.5 to 2 pH units. The enzyme is very stable at 40°C. in acid solution.

Lysozyme.—Smolens & Charney (112) have prepared antilysozyme by injecting crystalline lysozyme, made from egg white, into rabbits. A rather large proportion of the injected rabbits failed to respond. The immune serum of a few rabbits was capable of inhibiting the lysis of *Micrococcus lysodeiticus* by lysozyme.

Mechanism of insulin action.—Price, Cori & Colowick (113) have claimed that hexokinase, the enzyme which catalyzes the formation of glucose-6-phosphate, is inhibited by fractions from the anterior pituitary and that this inhibition is abolished by insulin. Broh-Kahn & Mirsky (114) could not regularly repeat this experiment but obtained fluctuating action by pituitary extracts. They suggest that the diabetogenic factor from the pituitary may be either extremely labile or else difficult to extract. Furthermore,

Broh-Kahn & Mirsky find that a substance causing hexokinase inhibition can be extracted from spleen. With this substance the reversal of inhibition by insulin addition is most inconstant. The authors find that if rats are rendered diabetic by alloxan, extracts of their muscles display a normal hexokinase activity and this is not affected by insulin. The conclusion is that disorders of glucose metabolism are not necessarily due to an inhibition of hexokinase activity. Colowick, Cori & Slein (115) have found that the hexokinase activity of extracts from the muscles of alloxan diabetic rats can be inhibited by adding adrenal cortex extract. Out of thirty extracts fifteen showed an inhibition of from 20 to 76 per cent, while fifteen inhibited 14 per cent or less. The authors found that the hexokinase of normal rat muscle or beef brain is not inhibited by adrenal cortex extract, but can be inhibited 30 to 75 per cent by further addition of 1 to 7 mg. of a protein fraction from the anterior pituitary. About 50 μ g. of insulin are found to counteract the effect of 0.1 cc. of adrenal cortex extract on diabetic muscle extracts. Insulin which has stood for three hours at 37°C. in 0.05 *N* KOH is inactive. The authors believe that the adrenocortical hormone is of pituitary origin. Butler *et al.* (116) find that, contrary to popular belief, trypsin has no appreciable destructive action upon insulin. Pepsin destroys insulin at pH 2, however, and chymotrypsin does at pH 8.

Papain.—Hoover & Kokes (117) have found that the optimum pH for the action of papain on casein is 5. It hydrolyzes benzoyl-argininamide, carbobenzoxy-L-isoglutamine and hippurylamide at the relative rates of 20:8:1. The optimum pH for hydrolysis of benzoylargininamide was approximately 5.5 and for the other two substrates 5.0.

The pectin methylesterase (pectinol or pectase) from mold cultures has a pH-activity curve different from that of higher plant pectin methylesterase, according to the findings of McColloch & Kertesz (118). Differences were observed also with respect to temperature coefficients, energies of activation, rates of thermal inactivation, resistance to ethanol and resistance to dialysis.

Hills & Mattern (119) have observed that gel formation is not a true measure of the activity of tomato pectase (pectin methylesterase), but that acid formation is. They find that the initial 40 per cent of the reaction is of zero order. The enzyme has a broad pH optimum with a maximum at 7.5. Addition of salts is necessary

for maximum activity. The optimum concentration of sodium chloride at pH 7.5 is 0.05 *M*. The enzyme is most stable at pH 4.0. It is inactivated at 70°C. at pH 6.5 in thirty minutes. Between 20 and 30°C. the temperature coefficient is 1.52.

Penicillinase.—LePage, Morgan & Campbell (120) have prepared penicillinase from the organism N.R.R.L. 569 and have purified it by adsorption on Hyflo Super-Cel, followed by elution with 0.075 *M* ammonia. The enzyme was then fractionated with ammonium sulfate. It is extracellular. Penicillinase of high activity has been obtained by Housewright & Henry (121) from *Bacillus aureus* grown in corn steep medium and also on a semisynthetic medium. The enzyme was purified by adsorbing on Filter-Cel with subsequent elution. Penicillinase is employed for the purpose of avoiding false negative results in culturing bacteria contaminated with penicillin. The authors describe (122) a manometric method for the estimation of penicillinase activity in which the hydrolysis of penicillin to form a free carboxyl group of p*K* 4.7 is catalyzed. The evolution of carbon dioxide from bicarbonate buffer is followed in the Warburg apparatus. The method was employed by Foster in 1945 (123).

Henry & Housewright find that their penicillinase inactivates penicillins G and X with equal rapidity but F and K at somewhat slower rates. The enzyme is highly specific. At 36°C. the optimum activity was at approximately pH 7.2. The temperature optimum was at 36°C., and at 46°C. the enzyme was rapidly inactivated. It is susceptible to oxidation but resistant to reduction. It does not contain copper or iron and requires neither amino nor sulfhydryl groups. It has been obtained from cultures of *Bacillus cereus* N.R.R.L. by Morgan & Campbell (124) who describe a method for purification. Penicillinase inhibitors (sodium benzoate and sodium sulfanilate) are reported upon by Reid, Felton & Petroff (125). The inhibitors are of value because they prevent the destruction of penicillin by the penicillinase of intestinal bacteria. Furthermore, the administration of penicillin together with sodium benzoate, sodium sulfanilate, bromsaligenin or certain other drugs helps to maintain the level of penicillin in the blood.

Peptidases of skin.—Fruton (126) has found that saline extracts of rabbit skin contain several proteolytic enzymes. One of these hydrolyzes L-leucylglycylglycine at pH 7.8 and does not require activation by manganous ions or by cysteine. This enzyme has

been named "dermopeptidase." It is not identical with leucine aminopeptidase, which is also present in the skin extracts. Its activity is retained upon dialysis, while that of leucine aminopeptidase is lost. Prolidase is present in the aqueous extracts of skin, but carboxypeptidase is absent.

L-Peptidases.—Zamecnik & Stephenson (127) have devised a manometric method to determine the activity of L-peptidase (catheptic enzyme). The substrate used is carbobenzoxy-L-glutamyl-L-tyrosine. The tyrosine which is set free is acted on by a decarboxylase and the volume of carbon dioxide thus formed is read in the manometer. The original substrate is not attacked by the decarboxylase. Using this method Frantz & Stephenson (128) have found that the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by catheptic enzymes follows a biphasic curve with zero order kinetics early in its course.

Roulet & Zeller (129) have also devised a method for the estimation of L-peptidases in which the amino acids liberated are subjected to the action of the L-amino acid oxidase of viper venom and the uptake of oxygen is followed in the Warburg apparatus. By the use of this method Roulet & Zeller have demonstrated the presence of L-peptidases in four human pathogens, one bovine pathogen and two nonpathogens. The peptides employed were glycyl-L-tyrosine, glycyl-L-leucine, L-leucylglycine and L-leucylglycylglycine. As is the case with other bacterial peptidases, 0.0001 *M* manganous salts caused activation. This was true especially for the hydrolysis of the tripeptide.

D-Peptidases.—In connection with the dubious claim of Kögl & Erxleben (130) that malignant tissue contains amino acids possessing a D-configuration, much research was carried out in Germany on D-peptidases. Waldschmidt-Leitz & Mayer (131) found that D-peptides were hydrolyzed by carcinoma serum more rapidly than by normal serum, the substrate being D,L-leucylglycine. Waldschmidt-Leitz, Hatschek & Hausmann (132) advanced the hypothesis that the D-peptidases present in malignant tumors act to protect the organism. According to Bamann & Schimke (133) the s-peptidases of sprouting plants are activated by manganous ions. They found (134) an increased concentration of D-peptidases in human malignant tissues.

Herken & Erxleben (135) devised a sensitive method for the determination of D-peptidase activity which depends upon the

employment D-amino acid oxidase and the action of the latter upon the D-amino acids set free by D-peptidases. Since L-amino acids will not react with D-amino acid oxidase one may employ racemic peptides in this procedure, if D-peptides are not available. In another paper Herken & Merten (136) reported upon the hydrolysis of the D-tripeptides, D-alanylglycineglycine, D-valylglycylglycine and D-leucylglycylglycine by serum; the results obtained were not uniform.

Ahlström, von Euler & Högborg (137) found that certain carcinoma sera have the ability to split D-peptides but do not believe that the presence of this enzyme in serum can be employed in the diagnosis of cancer. They reported that the procedure of Herken & Erxleben (138), wherein D-amino acid oxidase is employed, is a worthwhile supplement to older methods; however, they observe that kidney extract employed as a source of D-amino acid oxidase contains a D-peptidase. Addition of manganous salts activated various sera to different extents.

Phenolsulfatase.—Huggins & Smith (139) have employed *p*-nitrophenyl sulfate for the colormetric determination of phenolsulfatase. The enzyme was found in greatest concentration in the liver, next in the adrenals. Traces were present in human serum and urine. The pH optimum was at 6.19 in 0.5 *N* acetate at 37°C.

Phosphatases.—Thoai, Roche & Sartori (140) have obtained crystalline alkaline phosphatase from autolyzed beef liver, treated according to the method of Albers & Albers (141). The solution was fractionated with acetone. The product, on standing for several days at 6°C., crystallized. The enzyme could be recrystallized by adding forty to fifty volumes of acetone to one hundred volumes of enzyme solution and then dissolving the precipitate in a very small volume of dilute alkali. The crystalline material possesses an intense phosphomonoesterase activity at pH 9.2 and a slight pyrophosphatase activity. The "turnover number" is calculated to be somewhat lower than that of the crystalline zymohexase (aldolase) of Warburg & Christian (142). From a photograph, the phosphatase crystals appear to the reviewer to be needles in branch-like clusters.

Lehmann-Echternacht (143) obtained a very active nucleotidase from calf intestinal epithelium, 350 mg. of which completely dephosphorylated 100 gm. of ribonucleotide in twelve hours. The enzyme preparation had practically no action on polynucleotides and no action on nucleosides. It dephosphorylated 3- and 5-nucleo-

tides, glycerophosphate, monophenyl phosphate and phosphocreatine about equally well and acted also upon pyrophosphate. Diphenylpyrophosphate, diphenylphosphate and *p*-chloroaniline phosphoric acid were practically unattacked.

Lundquist (144) finds that the function of the acid phosphatase which occurs abundantly in human semen is to split phosphorylcholine into phosphate and choline. The phosphatase originates in the prostate. Andersch & Szczypinski (145) have determined serum acid phosphatase at pH 4.8 by the use of *p*-nitrophenylphosphate. The *p*-nitrophenol set free by the enzyme was determined colorimetrically after adding alkali.

The alkaline phosphatase of serum, according to Dalgaard (146), is increased in starved rats with obstructive jaundice.

Alkaline phosphatase has been demonstrated histochemically in decalcified dental and osseous tissue by Greep, Fischer & Morse (147). The decalcification was accomplished at pH 4.8 to 5.0 by using citrate buffer and formic acid. After incubating sections with glycerophosphate for three hours at 37°C., a visualization of enzyme action was brought about by the method of Gomori (148). The development of alloxan diabetes in rats is found by Cantor, Tuba & Caskey (149) to be accompanied by an increase in serum alkaline phosphatase activity. Administration of insulin produces a decrease in both acid and alkaline phosphatase. Winnick (150) finds that alkaline phosphatases from various sources readily hydrolyze phosphocreatine. This action is enhanced by the presence of magnesium ions. In view of this finding it appears possible that specific phosphoamidases, as postulated by Waldschmidt-Leitz, (151) may not exist. Alkaline phosphatase was found by Burgen & Lorch (152) to be inactivated strongly by alloxan. The inhibitory action of the alloxan appears to reside in the free 5-hydroxyl group. The inhibition is reversible, noncompetitive, and unaffected by magnesium ions.

Folley & Greenbaum (153) have noted changes in the arginase and alkaline phosphatase content of the mammary gland of the rat during pregnancy, lactation, and mammary involution.

Abul-Fadl & King (154) find that red blood cells contain roughly one hundred times as much acid phosphatase as does serum. This red cell phosphatase is not readily inactivated by formaldehyde, whereas prostatic phosphatase is readily inactivated.

Roche, Thoai & Roger (155) have dialyzed dog intestine phos-

phomonoesterase against water, cyanide or diethyldithiocarbamate at pH 6.0 and 37°C. until the enzyme was inactivated. The product was only slightly activated by magnesium ions but was very strongly activated by amino acids or polypeptides. Contrary to Hove, Elvehjem & Hart (156) zinc ions in the presence of amino acids had no activating action.

Acid phosphatase has been demonstrated in the mast cells of the rat by Montagna & Noback (157) using a slight modification of the method of Gomori (158) and of Wolf, Kabat & Newman (159). "The enzyme is localized in the mast cells in the form of discrete, coarse cytoplasmic granules, brown to black in color" (157).

Marsh & Drabkin (160) find that in cases of hyperglycemia there occurs an increase of both acid and alkaline phosphatases of the kidney. Phlorhizin inhibits both of these phosphatases *in vitro* and *in vivo*.

Adrenalectomy was found by Kutscher & Wüst (161) to lead to a decrease of both kidney and small intestine alkaline phosphatase in the guinea pig. The authors have concluded that some constituent of the adrenal cortex is required as the active group in alkaline phosphatase. Possibly this substance is a steroid.

Thoai (162) has found that the mushroom, *Lactarius deliciosus*, contains a pyrophosphatase with a pH optimum of 3.8 to 4.0 and a second pyrophosphatase with a pH optimum at 5.8 to 6.4. The mushroom contains a nondialyzable inhibitor which inactivates the second phosphatase. The inhibitor was separated through adsorption upon aluminum hydroxide. As substrates for these phosphatases Thoai employed sodium pyrophosphate and potassium diphenylpyrophosphate.

Von Euler, Hahn & Saluste (163) have investigated the action of nucleophosphatases of calf intestinal epithelium and horse kidneys upon various desoxynucleic acid and yeast ribonucleic acid preparations. They give methods for determination of nucleophosphatase and β -glycerophosphatase activity and give definitions of enzyme units in terms of inorganic phosphate set free. Methods are described for purifying nucleophosphatase and the effect of dialysis and electrophoresis are discussed, as well as the action of intestinal nucleophosphatase on various substrates. Zittle (164) states that the enzyme preparation from calf intestinal mucosa contains phosphodiesterase, phosphomonoesterase and a deami-

nase. The enzymes appear to be protein-polysaccharide complexes and are precipitated only by complete saturation with ammonium sulfate. If borate is present they are more easily salted out than in the absence of borate. Both phosphodiesterase and phosphomonoesterase (alkaline phosphatase) were found to be approximately 50 per cent inhibited by 0.01 *M* borate. Adenosine deaminase was not inhibited. Successive action upon calf thymus deoxyribonucleic acid of specific nuclease (165) and of phosphoesterase from calf intestinal mucosa was found by Zittle (166) to liberate four secondary phosphoric acid groups per tetranucleotide residue. In the absence of the specific nuclease the hydrolysis proceeded slowly to the extent of 70 per cent, but with both enzymes present hydrolysis was both rapid and complete. Zittle (167) has found that phosphodiesterase from calf intestinal mucosa completely hydrolyzes ribonucleic acid, liberating all of the phosphoric acid. This liberation of acid was followed manometrically through the production of carbon dioxide from carbonic acid—bicarbonate buffer. The enzyme was prepared by treating calf intestinal mucosa with trypsin according to Schmidt & Thannhauser (168). Zittle, Wells & Batt (170) have investigated the inhibition of the phosphoesterase of calf intestinal mucosa by 0.002 *M* sodium arsenate. It was found that the breakdown of both the diesters of phosphoric acid and the monoesters is inhibited, but the inhibition of the latter greatly exceeds that of the former. The inhibition by arsenic is not due to competition with the substrate.

Citrus fruit phosphatases have been investigated by Axelrod (169). He finds orange juice to contain a phosphomonoesterase but no diphenylphosphatase. The orange juice also was found to hydrolyze polyphosphates, including $\text{Na}_6\text{P}_4\text{O}_{13}$.

Pinguinain.—Asenjo & de Fernandez (171) have investigated the properties of the proteolytic enzyme pinguinain from *Bromelia pinguin* L. It resembles papain.

Phospho-enol transphosphorylase.—Kubowitz & Ott (172) have obtained phospho-enol transphosphorylase in crystalline form, using human thigh muscle as starting material. They have also purified human lactic acid apodehydrogenase.

Protaminase.—Portis & Altman (173) have devised a method for the estimation of protaminase activity through measurement of the quantity of arginine set free from salmine sulfate. While pepsin set free no arginine, crystalline trypsin and crystalline

chymotrypsin liberated one-half of the arginine present in salmine. The authors employed various procedures, but were unable to separate proteolytic activity from protaminase activity. It is probable that protaminase is identical with chymotrypsin.

Protease of pancreas.—Keith, Kazenko & Laskowski (174) have obtained a crystalline protein from the pancreas which has proteolytic properties after incubation with trypsin, or with purified enterokinase. It is not identical with chymotrypsinogen.

Purine enzymes as quantitative reagents.—Kalckar (175) has described the preparation of various enzymes which act upon purines and which he employs for the differential spectrophotometric determination of hypoxanthine, inosine, xanthine, guanine, guanosine, and uric acid (176) and also for the determination of adenosine and adenosine phosphates (177). The enzymes in question are adenosine deaminase, adenylic deaminase, adenylypyrophosphatase, xanthine oxidase, uricase, nucleoside phosphorylase and guanase.

Ribonuclease.—According to Schmidt and co-workers (178) yeast ribonucleic acid is completely resistant to prostatic phosphatase. However, if first hydrolyzed by sodium hydroxide or by ribonuclease, yeast ribonucleic acid is readily attacked by prostatic phosphatase and inorganic phosphate is split off. Hydrolysis by sodium hydroxide differs, however, from hydrolysis by ribonuclease, inasmuch as the preliminary sodium hydroxide treatment allows all of the phosphate to be split off through action of prostatic phosphatase, while preliminary treatment by ribonuclease allows only 25 per cent of the phosphate to be split off.

Chantrenne, Linderstrøm-Lang & Vanderdriessche (179) attribute two functions to ribonuclease: (a) as a depolymerase; (b) as a phosphatase. The depolymerization of ribonucleic acid causes an increase in volume, while the phosphatase action, whereby tetranucleotides are formed, causes a decrease in volume.

Staphylocoagulase.—Walker, Schaffer & Derow (180) have found that staphylocoagulase, the substance given off from cultures of *Staphylococci*, is destroyed by the action of pepsin and of trypsin. This enzyme, which coagulates plasma and which is extremely heat-stable, had been shown previously to be destroyed by trypsin [Walston (181)].

Synthesis by enzymes.—Ratner (87) has obtained from beef liver a soluble enzyme which catalyzes the anaerobic formation of

arginine and malic acid from citrulline and aspartic acid in the presence of magnesium ions, catalytic amounts of ATP, and phosphoglyceric acid as an ATP generator. Aspartic acid may be replaced by a mixture of glutamic and oxaloacetic acids, since under these conditions transamination takes place.

Thoai, Roche & Danzas (182) claim to have extracted from dog intestine and from sweet almonds a natural activator for the synthesis of esters by phosphatase. The substance is very labile and autolysis must be reduced to a minimum. In its presence synthesis occurs very rapidly, but the final equilibrium point is not changed. The activator is precipitated by acetone and by lead acetate and is set free from the latter by hydrogen sulfide. It dialyzes at 37°C. but not at 4°C. It activates both alkaline and acid phosphatases.

Hehre & Hamilton (183) have observed that bacteria of the *Neisseria* genus synthesize an amylopectin-like polysaccharide from sucrose. The product is rapidly digested by salivary amylase. The name "amylosucrase" is suggested for the enzyme system. Apparently glucose-1-phosphate does not participate in the synthesis as an intermediate. The authors suggest that the reaction is: $n \text{ sucrose} \rightarrow (\text{polysaccharide})_n + n \text{ fructose}$.

Hassid, Doudoroff & Barker (184) have synthesized several more disaccharides, using sucrose phosphorylase.

Borsook & Dubnoff (185) have demonstrated the synthesis of hippuric acid by liver homogenate, starting with benzoic acid and glycine. If more than traces of product are to be obtained it is necessary to homogenize the liver in the presence of the substrates. The yield was found to be doubled when adenylic acid and α -ketoglutaric acid were added. It is assumed that oxidation of the α -ketoglutaric acid resulted in the formation of ATP which furnished the free energy needed for the synthesis of the hippuric acid.

Speck (186) has found that glutamine is formed when glutamate and ammonia are added to isotonic Waring blender dispersions of fresh pigeon liver. The best yields were obtained when the digests contained oxygen, cytochrome-c, coenzyme I, oxaloacetate or citrate, phosphate, potassium ions, and magnesium ions. The addition of ATP did not accelerate the synthesis of glutamine.

Menne (187) has discovered that myosin possesses the property of an apoenzyme for the synthesis of creatine. If a phosphate extract of muscle, presumably containing a coenzyme, is added to

myosin, the preparation is capable of converting either histidine or arginine into creatine. Myogen, globulin X, serum albumin, serum globulin and fibrinogen were found to be incapable of taking the place of myosin.

Borsook & Dubnoff (188) have investigated the synthesis of urea in liver homogenate. They added to the homogenate L-ornithine, ammonia, L-glutamate, oxaloacetate, and ATP. In the absence of oxygen there was no synthesis of urea. Sodium arsenate, 0.0036 *M*, also prevented synthesis.

Altman & Evans (189) have demonstrated a synthesis of coenzyme II when dialyzed extracts of acetone-pigeon liver were treated with ATP. Addition of nicotinamide was found to increase the yield. A maximum yield was obtained by adding nicotinamide, ribose, and ATP. The synthesis was inhibited by 10^{-4} *M* sodium cyanide and 10^{-3} hydroxylamine, but not by iodoacetate or fluoride.

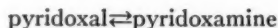
Schreiber (190) has investigated the effect of concentration of glycerol upon the synthesis of fat by various seed lipases. The optimum synthesis occurred with 88 to 90 per cent of glycerol when the lipases of *Linaria dalmatica*, *Linaria macedonica*, *Linaria striata* and *Loasa vulconica* were employed. With lipases from *Baccharis cordata* and *Glaucium luteum* the optimal concentration was about 80 per cent, while for the lipase from *Ricinus communis* *Zanzibarensis* it was only 55 to 60 per cent.

Thiamine-inactivating factor.—Wikén & Ågren (191) have employed the *Phycomyces* assay method, as modified by Wikén, to determine the activity of the thiamine-destroying enzyme in Swedish fish. The viscera of carp, roach, ide, rudd, *björkna* and mackerel contained considerable amounts of the enzyme. The enzyme was present also in the viscera of cod and "asp." The nervous and muscle tissues of the rudd were rich in the enzyme.

In a later paper Wikén & Ågren (192) state that, using the *Phycomyces* method, none of the thiamine-destroying enzyme could be found in tissue extracts of the calf, cow, pigeon, or toad. Viscera and muscles of the bream contained the enzyme in considerable amounts. Boiling for five minutes destroyed the enzyme.

Transaminase.—Ames, Sarma & Elvehjem (193) found that the hearts and kidneys of rats on a vitamin B₆ deficient ration contained only 40 per cent as much transaminase as did those organs from rats on diets adequate with respect to vitamin B₆.

The purification of transaminase from pig heart has been described by Schlenk & Fisher (194). The authors are inclined to believe that their product is of high purity. The prosthetic group was found to be very firmly bound to the apoenzyme. Silver and mercuric ions caused inhibition. The purified enzyme was found to be stable at 60° for one hour when in solution. Purified pig heart transaminase usually yielded pyridoxal, but tests sometimes showed pyridoxamine to be present. Possibly both of these substances were present. Schlenk & Fisher propose that the enzyme acts through the reaction:



Ormsby, Fisher & Schlenk (195) describe a test where diazotized sulfanilic acid gives an orange color with pyridoxine, orange to pink with pyridoxamine and yellow with pyridoxal.

A survey of the transaminases in plants has been made by Leonard & Burris (196). They found very active glutamic-aspartic and glutamic-alanine transaminases in wheat germ.

O'Kane & Gunsalus (197) have shown that the aspartic-alanine transaminase of Kritzmann (198) is an artifact. A mixture of purified glutamic-alanine transaminase, glutamic-aspartic transaminase, pyridoxal phosphate and glutamic acid functions as if it were an aspartic-alanine transaminase; the glutamic acid acts as a connecting link.

O'Kane & Gunsalus (199) have resolved pig heart glutamic-aspartic transaminase into apoenzyme and coenzyme. The apoenzyme was obtained in a higher state of purity than that previously attained with the holoenzyme. Pyridoxal phosphate serves as the coenzyme. The Michaelis dissociation constant of the enzyme, calculated by the Lineweaver-Burk method (200) was 1.5×10^4 moles per liter. Karrer & Viscontini (201) have found that crystalline pyridoxyl-acetal phosphate did not act as the coenzyme for the glutamic-aspartic transaminase of *Streptococcus faecalis*.

Tryptophanase.—Continuing the earlier work of Happold & Hoyle (202), Dawes, Dawson & Happold (203) obtained cell-free tryptophanase from *Escherichia coli* and showed it to consist of a dialyzable and nondialyzable portion. Later they found (204) that the apoenzyme of tryptophanase can be reactivated by pyridoxal phosphate, riboflavin, or coenzyme I. A mixture of all three gave a maximum effect. Wood & Gunsalus (205) also find that trypto-

phanase from dried cells of *Escherichia coli* requires pyridoxal phosphate. In a cell-free system no oxygen was taken up. The reaction was retarded by accumulation of indole and was inhibited by cyanide. Tryptophanase is superficially similar to the *Neurospora* enzyme which synthesizes tryptophane from indole and serine. Wood, Gunsalus & Umbreit (206) state that the tryptophanase prepared from *Escherichia coli* breaks tryptophane down into indole, pyruvic acid, and ammonia. Neither alanine nor serine was formed.

On the contrary, Dawes, Dawson & Happold (207) report that alanine is formed although serine is not.

Xanthine oxidase.—Polonovski and co-workers (208) find that xanthine oxidase is set free from fat globules of cow's milk upon cooling to 15°C. It is set free also by various detergents.

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CHEMISTRY OF THE CARBOHYDRATES¹

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SUGAR INTERCONVERSIONS

Goto (1) studied the transformation of glucose in aqueous alkaline solutions in the presence of D₂O and H₂O¹⁸. At 25°, 85 per cent of the glucose was transformed to fructose after eighteen days, two atoms of oxygen being replaced. Exchange of carbon-bound hydrogen was not observed at 25°, but it occurred in ten days at 55°.

Stetten & Stetten (2) concluded that biological conversion of inositol to glucose occurred when *meso*-inositol, containing carbon-bound deuterium, was fed to a phlorhizinized rat and significant amounts of deuterium were demonstrated in the urinary glucose.

The possible role of inositol as a reserve carbohydrate and as an intermediate in the biological transformation of one hexose into another, as well as the purely chemical transformation of glucose to a derivative of inositol, has been discussed by Fischer (3).

Percival & Duff (4) raised the question whether sugar interconversions in nature might not proceed through α,β -anhydrides, which could arise from suitably substituted sugar sulfates, and which commonly undergo hydrolytic scission accompanied by Walden inversion at one of the carbon atoms involved.

SUGAR ALCOHOLS AND THEIR ANHYDRIDES

According to older publications, β -sedoheptitol and its enantiomorph, D-gulo-L-talo-heptitol, fail to exhibit any measurable rotatory power. A reinvestigation by Merrill *et al.* (5) revealed that both pure heptitols do possess rotatory power in various solvents.

¹ The period essentially covered in this review extends from January, 1946 to August, 1947.

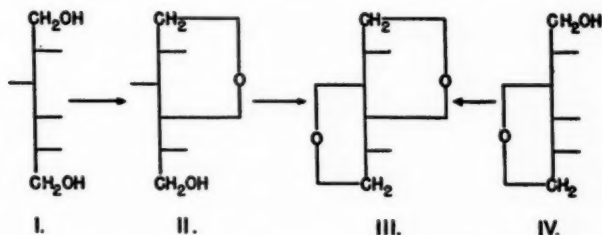
² Present address: National Institute of Health, Bethesda 14, Maryland.

Thus, the enantiomorphous character of both heptitols was firmly established, and β -sedoheptitol is L-gulo-D-talo-heptitol.

The products formed by the electroreduction of D-glucose under alkaline conditions have been investigated by Wolfrom *et al.* At pH 7 to 10, in addition to D-glucitol (sorbitol), 2-desoxy-D-glucitol (5 per cent) and D-mannitol (1 per cent) were isolated. This finding is in harmony with an enolic mechanism for sugar interconversion in alkaline media. *keto*-D-Fructose is assumed to be the precursor of the 2-desoxy-glucitol, several derivatives of which were prepared (6). When the reduction was performed at pH 10 to 13, 1-desoxy-D-mannitol (D-rhamnitol) could be isolated in addition to the alcohols just enumerated (7). Allitol, a 2-desoxy hexitol of unknown configuration (thought to be 2-desoxy-D-allitol), and a pentitol of unknown structure were also isolated. An attempt to synthesize 2-desoxy-allitol from *keto*-D-psicose pentaacetate via the dithioacetal resulted in the production of 1,6-(*erythro*-3,4)-hexanetetrol (2, 5-didesoxy-allitol) but hydrogenation of the *keto*-psicose acetate gave allitol, isolated as the 2,4:3,5-dimethylene derivative in small yield. 1-Desoxy-D-glucitol and several of its derivatives have been prepared from 1-desoxy-*keto*-D-fructose tetraacetate (8). 2-Desoxy-D-allitol has recently been prepared from 2-desoxy-D-allose (9); the constants recorded for this substance are at variance with those of Wolfrom's 2-desoxyhexitol. Although predicted as a possible component of the reduction mixture (7), 1-desoxy-D-glucitol has so far resisted isolation (8). Finally, the isolation of the hitherto unknown D,L-glucitol from the reduction mixture has been reported. It was suggested that allitol and D,L-glucitol might arise by enolisation proceeding along the chain. The alternative possibility of (cathodic) dehydrogenation of any of the hydroxyl groups and subsequent reduction of the newly formed keto group was also considered (10). Oxidation of 2-desoxy-D-glucitol by *Acetobacter suboxydans* gave 5-desoxy-L-sorbose (11).

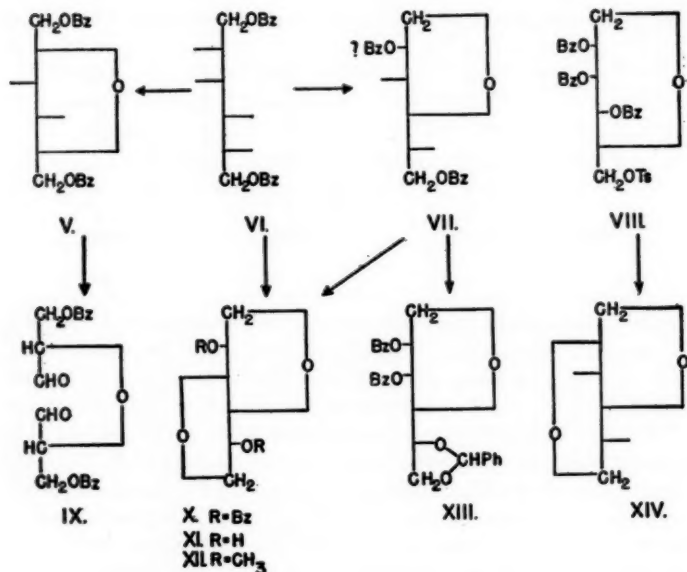
The work of Wiggins, Hockett, Goepp, and their respective associates shows that anhydriization of sugar alcohols with acidic reagents results in the preferential formation of 1,4-anhydro rings. Thus, sorbitol (I) gave 1,4-anhydro-D-glucitol ("arlitan") (II) (12), the structure of which follows from its degradation to dimethyl-L-threic acid, and from its oxidation by lead tetraacetate, whereby one mole of formaldehyde resulted. It is not identical

with 3,6-anhydro-D-dulcitol (13) which indicates that anhydri-
zation is not accompanied by Walden inversion on C-4. Further
anhydri-
zation of either 1,4-(II) or 3,6-anhydro-sorbitol (IV) gives
1,4:3,6-dianhydrosorbitol (isosorbide) (III) (14), also obtained on
saponification of the 1-tosyl-2,4,5-triacetyl derivative of IV with
sodium methylate (15). The dibenzoate of III is obtained by
heating 1,6-dibenzoyl-sorbitol with a trace of *p*-toluenesulphonic
acid in *sym*. tetrachloroethane (14) indicating migration of primary
acyl groups under these conditions. This led to a reinvestigation of



the structures of the three anhydromannitols obtained by Brigl &
Grüner (16) by similar treatment of 1,6-dibenzoylmannitol (VI).
The correct structures, mainly derived from lead tetraacetate
oxidation data, are 1,4-anhydro-mannitol 2,6(or 3,6)-dibenzoate
(VII) (Brigl's "2,4-anhydromannitol 1,6-dibenzoate"), 1,4:3,6-
dianhydro-mannitol (isomannide) 2,5-dibenzoate (X) (Brigl's
"2,4:3,5-dianhydromannitol 1,6-dibenzoate") (17), and 2,5-anhy-
dro-sorbitol 1,6-dibenzoate (V) (Brigl's "2,5-anhydro-mannitol
1,6-dibenzoate"). Formation of V which is oxidized by lead tetra-
acetate to the inactive dialdehyde IX without liberation of formal-
dehyde, implies Walden inversion on C-2 during anhydri-
zation (18). Benzalation of VII with zinc chloride as the condensing agent
is accompanied by acyl migration since the product is the diben-
zoyl benzylidene derivative XIII. Isomannide (XI) is not attacked
by lead tetraacetate and it is not identical with 1,4:2,6-dianhydro-
mannitol (neomannide) (XIV) obtained from 6-tosyl-tribenzoyl-
styracitol (VIII) on reaction with sodium methoxide (19). These
findings are in harmony with structure XI for isomannide, already
advanced by Wiggins (20) who prepared it by dry distillation of
1,6-dichloromannitol and the dimethyl ether XII by the action of
sodium methoxide on 1,6-dichloro-2,5-dimethyl-mannitol.

1,4:3,6-dianhydro-L-itol (isoidide) was obtained from L-itol as well as from isosorbide and isomannide (21). By catalytic dehydrogenation of either isohexide, ketonic functions were formed at C-2 or C-5, or both, and subsequent hydrogenation of the mixture gave isoidide, along with isosorbide and isomannide. The conversion of isosorbide to isoidide involves inversion of only one hydroxyl group, whereas in isomannide two hydroxyl groups must



change their configuration. Consequently, the yield of isoidide from isosorbide is much better than from isomannide.

Isosorbide and isomannide, which can easily be prepared from sucrose (22) are of considerable interest, since a number of derivatives have been prepared which have found application in a variety of preparations [cf. (22)]. It is interesting to note that in these dianhydro hexitols exchange of the secondary hydroxyl groups (as tosylates) for iodine and amino groups proceeds rather smoothly (23).

In the pentitol series, dehydration also results in 1,4-anhydro

ring formation. Treatment of xylitol with benzenesulfonic acid gave D,L-1,4-anhydro-xylitol (24).

In order to obtain 1,5-anhydro pentitols and hexitols, it is most convenient to start from the corresponding thiopyranosides and subject these to reductive desulfurization with nickel. Thus, 1,5-anhydro-xylitol (25) and 1,5-anhydro-D-arabitol (26) were obtained from the aryl 1-thiopentopyranosides. 1,5-Anhydro-D-glucitol (polygalitol) previously obtained from 1-thio-D-glucopyranose was obtained in a similar way from ethyl tetraacetyl-D-glucopyranosyl xanthate (27).

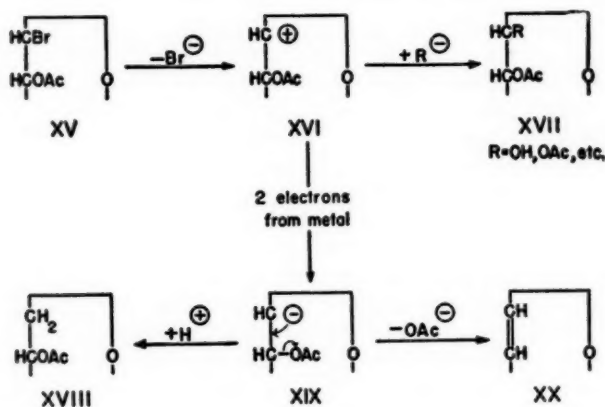
DESOXY SUGARS

2-Desoxy sugars.—The well-known method of Emil Fischer for the transformations of aldoses into glycals is still the most direct method for the preparation of 2-desoxy sugars, which may be obtained from the corresponding glycals by mild acid hydrolysis. A modification (28) of Fischer's method dispenses with the isolation of the acetobromo aldose and permits of isolation of the acetylated glycal in improved yield. However, the yields of glycal vary apparently depending on the structure of the aldose used [cf. (44)]. This seems to point to steric effects exerting an influence on the course of the reaction. The following mechanism might conceivably operate in the formation of a glycal from an acetobromo aldose:³ Ionization of the acetobromoaldose (XV) furnishes the cation XVI, which may subsequently either yield the neutral compound XVII, or the anion XIX. In the former case simple substitution at C₁ occurs, the extent of which will depend on the relative ratio of the reaction velocities XVI→XVII and XVI→XIX. Steric hindrance from the substituent on C₂ will favor the latter change. During the change XVI→XVII Walden inversion may occur. Alternatively, the cation XVI may be transformed to the anion XIX. At this point, again two reactions may occur, probably simultaneously: (a) the negative charge on C₁ in XIX gives rise to an electron displacement as indicated by the curved arrows, resulting in the ejection of an acetate ion from C₂, and formation of the glycal (XX), or (b) a proton satisfies the negative charge in XIX and the alditol anhydride XVIII is formed. Here also, the relative ratio of the velocities XIX→XX and XIX→XVIII will influence the

³ This mechanism was suggested by Prins and discussed by Iselin (96).

course of the reaction; even more predominantly so, since steric effects, which would operate in the change XIX→XVIII, should exert only a minor influence, due to the smallness of the entering proton. Theoretically, also the interaction of XVI and XIX to form a dimer is conceivable. That C₂ must carry an ionisable substituent if a glycal is to be formed is borne out by the fact that 2-methyl aldoses do not furnish glycals (29).

So far, compounds of the anhydro alditol type (XVIII) seem to have escaped detection. Consideration of steric factors might



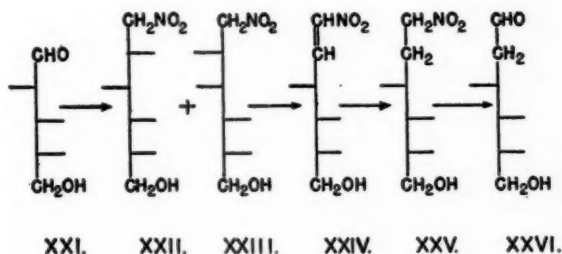
furnish an explanation for the varying yields of glycals obtained from different aldoses. An extension of the study of Scattergood & Pacsu (30) on models might prove useful.

Failure of Fischer's glycal method was recorded by Grob & Prins (31) in the case of 3-methyl-altromethylose. This led Jeanloz, Prins & Reichstein (32) to search for other methods for the preparation of 2-desoxy sugars, which resulted in the observation that certain 2,3-anhydro hexosides, such as (XXVIII), will react with sodium methylmercaptide to form 2-methylthiohexosides (XXVII) which in turn can be reduced to 2-desoxy hexosides (XXXI). If this reductive desulfurization is carried out with a large excess of nickel, 4,6-benzylidene groups, if present, are split off hydrogenolytically. If less nickel is employed, desulfurization without loss of the benzylidene group may be achieved (33, 34). The structures of the resulting desoxy hexosides were proved by

oxidative degradation to the corresponding methoxysuccinic acid (XXXII) (32, 33). Methylation of the free hydroxyl function proceeded well in the case of the 2-methylthio-altrose derivative (XXVII), but when the analogous 2-methylthio-idose derivative was treated with Purdie's reagents, the desired methyl ether could be isolated only in small yield (33). Anomalous methylation was also observed in another instance (35). The anomaly may be due to the reactivity of the sulfur atom, which is known to give rise to sulfonium bases.

Wiggins *et al.* (36) have recently made the observation that certain 2,3-anhydro aldoses react with anhydrous hydrochloric and hydrobromic acid to form mixtures of 2- and 3-halo-aldosides. It is clear that the 2-halo-aldosides may serve as intermediates for the synthesis of 2-desoxy aldoses.

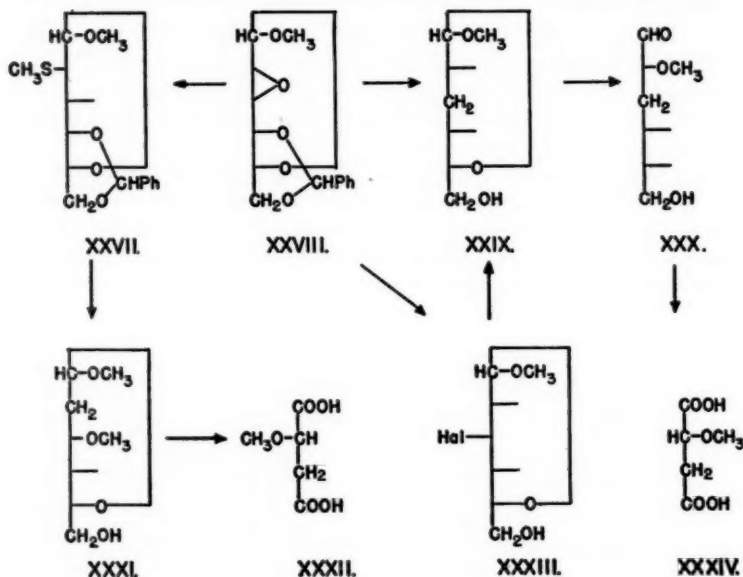
Another route to 2-desoxy sugars has been described by Sowden & Fischer (38). Condensation of an aldopentose (XXI) with nitromethane (39) yields a mixture of epimeric C-nitro alcohols (XXII and XXIII) which on acetylation and subsequent treatment with bicarbonate in a nonpolar solvent give the acetylated nitroolefin (XXIV). XXIV, on further treatment, yields the acetylated nitroolefin (XXV). XXV, on further treatment, yields the acetylated nitroolefin (XXVI).



This is partially hydrogenated to the 1-nitro-1,2-dideoxy-hexitol (XXV), the aci-sodium salt of which is hydrolysed to the 2-desoxy-hexose (XXVI) by sulphuric acid [cf. (39, 40)].

Further possible intermediates for the preparation of 2-desoxy-aldoses are the 2-alkylthio-aldoses described by Brigl & Schinle (41), and the 2-desoxy-aldehydic acids obtained by Wolfrom *et al.* (42). Attempts to exchange the 2-tosyloxy group in 2-tosyl aldoses for a thiomethyl group have been unsuccessful (43, 33).

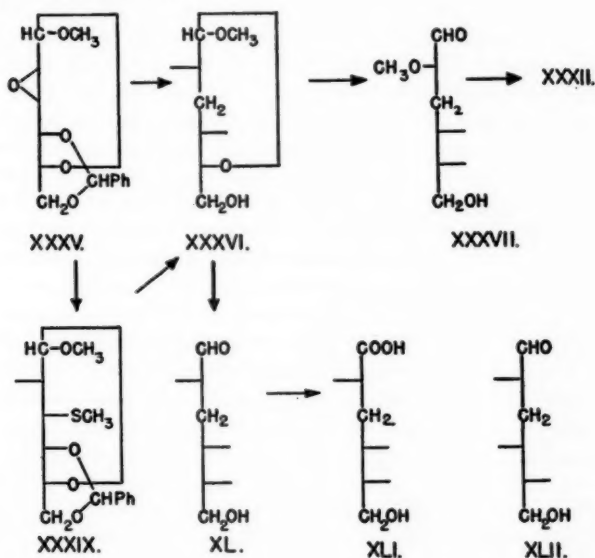
Of the methods discussed, those of Emil Fischer and of Sowden & Fischer have the advantage of general applicability. However, both involve the carbonyl carbon and the yields of 2-desoxy sugar are not always quite satisfactory. The methods involving 2,3-anhydro-aldosides are limited in applicability, since both 2- and 3-desoxy-aldosides may result as the final products, depending upon the structure of the molecule containing the ethylene oxide ring. In those instances where 2-desoxy-aldosides are formed, however,



these methods have the advantage of being applicable to glycosides and, as in the case of the mercaptide method, the over-all yields are generally good.

3-Desoxy sugars.—Little is known about this group of desoxy sugars, which so far have not been reported to occur naturally. The availability of 2,3-anhydro-aldosides has now led to the preparation of several 3-desoxy-aldosides. Irrespective of the configuration of the anhydro ring, hydrogenation of 2,3-anhydro-aldosides leads, in the instances investigated (35, 44, 45), to 3-desoxy sugars. However, on treatment with anhydrous hydrogen halides, or, in

certain instances, with sodium mercaptide, 3-halo- or 3-methylthio-aldosides may be obtained, from which the 3-desoxy sugars are obtained by catalytic reduction. Thus, the 2,3-anhydro-alloside derivative (XXVIII) gave methyl 3-desoxy- α -D-glucoside (XXIX) on hydrogenation (44). Proof of structure was furnished by benzalation, methylation and subsequent hydrolysis of XXIX, which furnished reducing 2-methyl-3-desoxy-D-glucose (XXX), oxidation of which yielded D(+)-methoxysuccinic acid (XXXIV). Wiggins *et al.* also obtained XXIX from XXVIII via the 3-halo derivative XXXIII (36).



The 2,3-anhydro-mannoside derivative (XXXV) (46) was hydrogenated to methyl 3-desoxy- α -D-mannoside (XXXVI) which was also obtained from XXXV via the 3-methylthio-altrioside derivative (XXXIX) (35). Benzalation, methylation and hydrolysis of XXXVI yielded 2-methyl-3-desoxy-D-mannose (XXXVII) which was oxidized to L(-)-methoxysuccinic acid (XXXII). Hydrolysis of XXXVI furnished syrupy 3-desoxy-D-mannose (*syn.* 3-desoxy-D-altrose) (XL), characterized by its *p*-nitrophenyllosazone. Oxidation of XL with bromine water gave

3-desoxy-D-mannonic acid (XLI) characterized by its phenylhydrazide.

Derivatives of 3-desoxy-D-idose (XLII) have been prepared from methyl 2,3-anhydro-4,6-benzylidene- β -D-talocide by the mercaptide method, and their structures proved (34).

In the pentose series, derivatives of 3-desoxy-L-xylose (*syn.* 3-desoxy-L-ribose) have been described (45). Methyl 2,3-anhydro- β -L-ribopyranoside was treated with sodium mercaptide, but, contrary to expectation, the principal product obtained by Mukherjee & Todd was methyl 3-methylthio- β -L-xyloside, which was reduced to the 3-desoxy-xyloside. When methyl 2,3-anhydro- α -L-ribopyranoside was reacted with sodium mercaptide and subsequently reduced, the 3-desoxy-xyloside was again the principal product, but in this case some methyl 2-desoxy- α -L-riboside may have been formed, since the crude reduction mixture gave a feeble Keller-Kiliani reaction and it consumed 0.3 moles of periodate per mole of methyl-desoxypentoxide. However, no 2-desoxy-ribose could be isolated, and the periodate titration value may have given an exaggerated estimate of the content of 2-desoxy-pentoxide in the mixture. It may be mentioned in this connection that methyl 3-desoxy- α -D-glucopyranoside (XXIX), which should not consume periodate, was found to be oxidized by this reagent, and no definite end-point could be determined (47). Anomalies of the Keller-Kiliani reaction have been recorded (32, 67).

The unexpected behavior of the 2,3-anhydroribopyranosides towards sodium mercaptide has been commented upon (34), and it was suggested that the nature of the product obtained from the interaction of 2,3-anhydropyranosides and sodium methoxide or mercaptide depends on the structure around C₅ in the anhydropyranoside molecule. In fact, the influence of substitution at C₅ on the course of ring scission in 2,3-anhydropyranosides is illustrated by the following examples: with alkaline reagents, both methyl 2,3-anhydro-4,6-benzylidene- α - and β -D-allopyranoside yield preponderantly derivatives of altrose (scission at C₂) (31, 48); similarly, methyl 2,3-anhydro-4,6-benzylidene- α -D-gulopyranoside (scission at C₂) as well as methyl 2,3-anhydro-4,6-benzylidene- α - and β -D-talopyranoside (scission at C₃) give derivatives of idose in almost quantitative yield (34, 49). This indicates that the influence of the configuration of the glycosidic methyl group is practically negligible, and the course of the reaction appears to be determined largely by the configuration of the ethylene oxide ring.

Methyl 2,3-anhydro-4,6-benzylidene- α -D-mannopyranoside follows the "rule" in that, with alkaline reagents, it gives derivatives of altrose almost quantitatively. In fact, neither with sodium methoxide (44) nor with ammonia (50) was it possible to detect the formation of derivatives of glucose. However, methyl 2,3-anhydro-4,6-dimethyl- β -D-mannopyranoside, in which the substitution around C₆ is much less "bulky," gave equimolecular quantities of altrose and glucose derivatives (51). With ammonia, methyl 2,3-anhydro-4,6-benzylidene- β -D-talopyranoside gave 63 per cent of the 3-amino-idose derivative, but from 1,6:2,3-dianhydro- β -talose 56 per cent of the 2-amino-galactose derivative was obtained (52). Here again there is a marked effect of the spatial structure around C₆ on the course of the reaction.

In the pentose series however, where the substituent on C₅ is hydrogen, methyl 2,3-anhydro- α - and β -L-ribose as well as methyl 2,3-anhydro- β -L-xylopyranoside furnished predominantly derivatives of xylose when treated with alkaline reagents (45, 53) and polarization of the oxide ring appears to be reversed as compared with the benzylidene anhydrohexosides. In this connection, it is interesting to note that whereas the 2,3-anhydro-alloside derivative (XXVIII) with alkaline reagents gives derivatives of altrose as the principal product, with acidic reagents derivatives of glucose are predominantly formed (36). Hence it would appear that not only steric conditions play a role, but that also the pH of the reaction medium is involved in determining the point of attack on the ethylene oxide ring. Attention is drawn to an excellent treatise on anhydro sugars by Peat (37).

6-Desoxy sugars.—In cases where the exchange of the primary tosyloxy group at C₆ for iodine proceeds with difficulty in acetone, better results may be obtained with acetic anhydride as the solvent (54). The use of acetonylacetone (171) instead of acetone obviates the sealed tube technique. The use of a moderate excess of diethylamine instead of alkali as acceptor for the hydroiodic acid formed in the catalytic reduction of the 6-iodohydrin to the 6-desoxy derivative is also recommended (55). Transformation of the 6-iodohydrin to the 6-thiuronium salt and subsequent reductive desulfurization has also been used for desoxylation at C₆ (56).

Desoxy sugars from cardiac glycosides.—An excellent review on this subject has been prepared by Elderfield (57). Recent contributions to this field are summarized below:

L-Fucose.—L-Fucose, although fairly widely distributed in na-

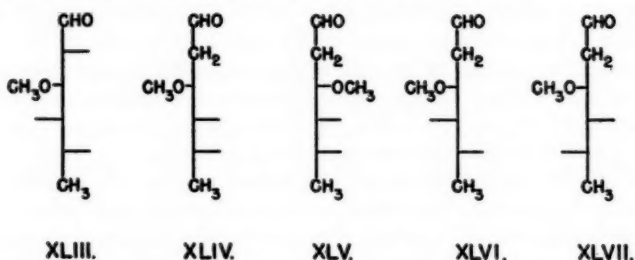
ture has not yet been found to occur in cardiac glycosides. Recently, Schmutz & Reichstein (58) isolated two new glycosides from the seeds of *Strophanthus sarmentosus*, designated sarmentoside A and B. Hydrolytic cleavage of sarmentoside A gave a reducing sugar, the phenylosazone of which appears to be identical with that of L-fucose. The melting point and specific rotation of authentic L-fucose phenylosazone were redetermined.

Digitalose.—Following definite establishment of the structure of this rare sugar (XLIII) by Schmidt *et al.* (59), its synthesis from D-galactose has been accomplished by Reber & Reichstein (60). Digitalose is probably a component of sarmentoside B (58).

Digitoxose.—Using the glycol method, Iselin & Reichstein (61) prepared this sugar from D-allomethylose, which in turn was prepared from L-rhamnose. The transformation of D-glucose to digitoxose via the 2,3-anhydroalloside (XXVIII) and using the mercaptide method was also realized (9).

Oleandrose.—From the synthesis of D-oleandrose (XLIV) (62) it follows that the natural sugar belongs to the L-series.

Cymarose.—This sugar (XLV) was synthesized from D-glucose via the anhydroalloside (XXVIII) in good over-all yield (63) confirming the structure established by Elderfield (64).



Diginose and sarmentose.—These rare sugars represent the remaining members of the 3-methyl-2,6-bisdesoxy-hexose group. On oxidation, diginose gave D (+)-methoxysuccinic acid (XXXIV) and acetic acid (65) and therefore must possess structure XLVI or XLVII. Comparison of its optical rotation with that of 2-desoxy-L-fucose (66) ($[\alpha]_D +55^\circ$ and -62° respectively; final values in water) would suggest that diginose may be 2-desoxy-digitalose (XLVI) leaving structure XLVII (or that of its enantiomorph) for sarmentose.

Other desoxy sugars.—In addition to the desoxy sugars mentioned above, the preparation of the following crystalline desoxyaldoses has been described; 3-methyl-2-desoxy-D-glucose (67), 2-desoxy-D-allose (9), D-rhamnose from methyl α -D-mannoside (55), D-fucose from methyl α -D-galactoside (43), L-idomethylose (68) and 3-methyl-D-altromethylose (31) from glucose. D-Altromethylose, also from glucose, was obtained in the amorphous state (69). Its preparation from D-allomethylose was also reported (70). D-Ribomethylose was obtained from D-allomethylose by Wohl degradation (71).

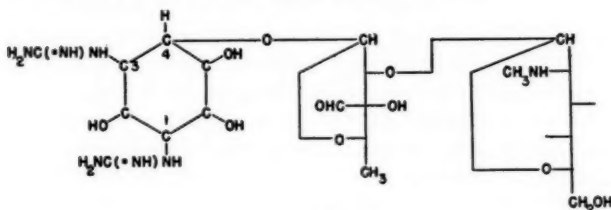
STREPTOMYCIN

The spectacular progress made in the chemistry of streptomycin is mainly due to the efforts of Folkers, Wintersteiner, Wolfrom, and their respective associates. Formula XLVIII has been advanced for this antibiotic by Folkers *et al.* (72). The pertinent data leading to this structure may be summarized as follows: Hydrolytic cleavage of streptomycin gave streptidine (73, 74) which was found to be one of the meso-forms of 1,3-diguanidino-2,4,5,6-tetrahydroxy-cyclohexane (75).

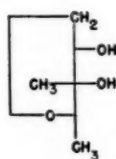
On methanolysis, streptomycin hydrochloride afforded methyl streptobiosaminide dimethylacetal (76) which was further cleaved to yield N-methyl-L-glucosamine (76, 77). This amino sugar was synthesized from L-arabinose, methylamine and hydrogen cyanide (77, 78). Mercaptolysis of streptomycin hydrochloride furnished ethyl thiostreptobiosaminide diethylthioacetal (76, 79) which was reduced to bisdesoxydihydrostreptobiosamine (79, 80), hydrolytic cleavage of which gave N-methyl-L-glucosamine and bisdesoxystreptose [bisdesoxy dihydrostreptonose (XLIX)]. Later (81) both anomeric forms of ethyl thiostreptobiosaminide diethylthioacetal were obtained, and since both furnish one and the same product on reductive desulfurization, bisdesoxy-dihydrostreptobiosamine, this is conclusive evidence for the glycosidic linkage between streptobiosamine and streptidine. Furthermore, in streptobiosamine, N-methyl-L-glucosamine is similarly linked to the streptonose moiety. Further transformations of streptomycin and streptobiosamine (82, 83) lend additional support to the structures advanced for these compounds.

Bisdesoxystreptose gives a complex with boric acid suggesting *cis*-configuration of the two hydroxyl groups. After oxidation with

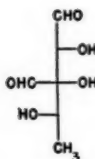
one mole of periodate, subsequent hydrolysis of the oxidation product, and treatment with phenylhydrazine, the osazone of diacetyl was isolated (80). Clearly, this fragment includes the methyl side chain and the carbon atoms 3 to 5 of XLIX. Treatment of streptobiosamine with phenylhydrazine yielded the phenylosazone of 4-deoxy-L-erythrose (84) formed from the same part of the streptonose moiety, since it is not obtained from dihydrostreptobiosamine. The isolation of this compound establishes that streptonose belongs to the L-series. Structure L for streptonose is compatible with the experimental data. However, its configuration (a or b) remains to be established.



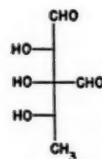
XLVIII.



XLIX.

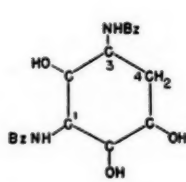


(a)



(b)

L.



LI.

The presence of a free carbonyl group in streptomycin is demonstrated by the formation of carbonyl derivatives (85, 86) and of a dihydroderivative (74, 87) in which the carbonyl group is reduced to hydroxyl. That the carbonyl is aldehydic follows from its reduction to a methyl group. Oxidation of streptomycin with bromine water (74) gave streptomycinic acid, indicating oxidation of the free aldehyde group in the streptonose moiety to carboxyl. Streptomycinic acid, and also dihydrostreptomycin, fails to produce maltol on treatment with alkali, a reaction characteristic for streptomycin (88) and indicative of the intact aldehyde group in the streptonose portion.

On acetylation, methyl streptobiosaminide dimethyl acetal and ethyl thiostreptobiosaminide dithioethyl acetal furnish tetraacetates. Since the glucosamine portion accounts for four acetyl groups, there is no chemical evidence for the free (tertiary) hydroxyl group in these compounds, but at the same time this result indicates that the hydroxyl group at C₂ of streptonose is engaged in the glycosidic link combining the streptonose and glucosamine portions. More rigid proof was furnished (89) when the rates of oxidation of methyl N-acetyl-dihydrostreptobiosaminide and of methyl N-acetyl-streptobiosaminide dimethyl acetal by periodate were compared. Only the former substance showed rapid consumption of one mole of periodate with concomitant formation of formaldehyde. A similar behavior was exhibited by the hydrochlorides of streptomycin and dihydrostreptomycin. Oxidation with 1,5 moles of periodate resulted in the formation of formaldehyde from the latter only. Thus, the 1,4-furanose ring demonstrated in streptobiosamine is also present in streptomycin.

The position of the linkage between streptidine and streptobiosamine was established (72) when heptabenzoylstreptidine, obtained from fully benzoylated streptomycin, was converted to heptabenzoyl-desoxystreptidine and hence to N,N'-dibenzoyl-desoxystreptamine (LI), which consumed one mole of periodate. Under comparable conditions, N,N'-dibenzoyl-streptamine consumed two moles of this reagent. This places the desoxy group, and with it the hydroxyl group involved in the glycosidic link, at C₄. Previously, the isolation of streptidine from the oxidation of streptomycin with excess periodate, and subsequent hydrolysis, had been reported (90). From this it was inferred that the glycosidic linkage probably was at C₆, although C₄ could not entirely be excluded, since 1,2,3,4-tetraacetyl-inositol is resistant to periodate oxidation (91).

Application of Hudson's isorotation rules to various anomeric derivatives of streptobiosamine (89) indicates that both glycosidic linkages in streptomycin are α -L. A method for the colorimetric determination of streptomycin in clinical preparations, urine and broth has been described (92). The present status of streptomycin in clinical medicine was reviewed by Hirschfeld & Buggs (93).

Streptomycin B.—Fried & Titus (94) reported the isolation of streptomycin B from streptomycin concentrates and Fried & Stavely (95) described its degradation to streptidine, streptobiosamine and D-mannose. It appears that streptomycin B is closely

related to streptomycin in that these fragments are linked glycosidically. The hitherto unknown α - and β -forms of ethyl tetraacetyl-1-thio-D-mannoside were obtained from the mercaptolysis of streptomycin B, and the β -anomer was prepared from mannose for comparison. Like streptomycin, streptomycin B yields a dihydro derivative, methanolysis of which, followed by acetylation, furnished methyl pentaacetyl- α -dihydrostreptobiosaminide and methyl tetraacetyl- β -D-mannopyranoside. The antibiotic activity of streptomycin B is from one fourth to one eighth of that of streptomycin.

DISACCHARIDES AND TRISACCHARIDES

Sucrose might arise by condensation of the tetraacetates of 1,3,4,6-D-fructofuranose and 2,3,4,6-D-glucopyranose. This reaction, using phosphorus pentoxide as the condensing agent, was re-investigated by Binkley & Wolf from (97). Chromatographic analysis of the reaction mixture revealed that only isosucrose octaacetate had been formed.

Esters of trehalose have been found among the lipid constituents of acid resistant bacilli. This led Willstaedt & Borggard (98) to prepare the 2,3,4,6,2',3',4',6'-octastearyl and 2,3,4,2',3',4'-hexastearyl esters of this disaccharide.

The structure of difructose anhydride II, which is obtained from inulin by the action of acid, was established by McDonald & Turcotte (99) as 2,1'-4,2'-di-D-fructofuranose anhydride.

Controlled hydrolysis of the dextran from *Leuconostoc dextranum*, followed by the removal of glucose by fermentation, acetylation of the residue, and chromatography, permitted the isolation of a disaccharide which, in view of the structure of the polysaccharide, can only be 6- α -D-glucopyranosido- β -D-glucose (100). When the product of enzymatic hydrolysis of the amylopectin from waxy maize was submitted to the same treatment, maltose octaacetate and a second crystalline compound were isolated, which differed from the above mentioned disaccharide and which probably is a trisaccharide.

Hassid and co-workers (101) prepared two new disaccharides from glucose-1-phosphate and L-arabinose by enzymatic synthesis. One of these is the first reducing disaccharide to be prepared from a hexose in this manner. The behavior of its osotriazole derivative towards periodate as well as the formation of 2,3,4,6-tetramethylglucose and 2,4-dimethyl-L-arabinose from the fully methylated

disaccharide indicate that it is 3-(α -D-glucopyranosido)-L-arabinopyranose. The second disaccharide, which is nonreducing, has a structure analogous to sucrose and is α -D-glucosido- β -L-arabinoside. Maltose-1-phosphate does not undergo polysaccharide formation when treated with potato phosphorylase (102). This is of theoretical importance, since it bears on the mechanism of the enzymatic formation of starch from smaller units.

The structure of melezitose has been further elucidated by Richtmyer & Hudson (103). On periodate oxidation no formaldehyde was formed (104), four moles of oxidant were consumed and two moles of formic acid liberated. These findings, combined with previous data on melezitose, establish its structure as 3-(α -D-glucopyranosyl)- β -D-fructofuranosyl-D-glucopyranoside. The nature of the second glycosidic link remains to be established. This problem was recently discussed by Hudson (105).

POLYSACCHARIDES

Starch.—In comparison with previous years, relatively few new investigations were conducted on this problem, the principal points of which have been elucidated. In a series of papers, Pacsu *et al.* (106) and Sutra (107) have taken up the old concept of the homogeneity of starch, however, without presenting convincing proof to support their hypothesis. Pacsu suggested that a small number of noncyclic hemiacetal linkages causes discontinuity in the α 1-4 chain. This view has been refuted by Hirst and co-workers (108).

A starch with entirely new properties has been found by Hilbert & MacMasters (109) in three varieties of garden type wrinkled seeded peas. By measuring the complex formed with iodine, and by fractionation, these authors found that the amylose content varied from 60 to 70 per cent, depending on the variety studied.

Studying the x-ray patterns of oriented filaments of amylose and alkali amylose, obtained from potato amylose acetate by de-esterification, Senti & Witnauer (110) found for alkali amylose an orthorhombic unit cell having $a_0 = 9.0$ Å, $b_0 = 22.7$ Å and $c_0 = 12.7$ Å, and containing twelve $C_6H_{10}O_5 \cdot (KOH)_x$ groups. The transformation of the alkali amylose structure to the V-structure is brought about by methanol, giving a fiber with a repeat period of 8 Å. Lateral spacings vary with moisture content. Extraction of alkali amylose with *t*-butanol modifies the lateral reflexions, and a monoclinic unit cell is obtained, the fiber period being 7.9 Å.

The screw-like structure of amylose, suggested by Hanes (111)

and Freudenberg (112) and verified for the solid amylose-iodine complex by French & Rundle (113) by means of x-ray diffraction has been studied for amylose in solution by Dombrow & Beckmann (114). The molecule has the shape of an ellipsoid of revolution, the dimensions of which may be calculated by viscosimetry, ultracentrifugation and diffusion. These methods furnish dimensions which are compatible with those of the helical model calculated from x-ray diffraction data.

Glycogen.—A method for the quantitative determination of glycogen involving colorimetry of its iodine complex was described by Jung (115) and adapted for micro-work by van Wagtenonk *et al.* (116). This method has been criticized by Morris (117) who showed that the color varies not only with the concentration of glycogen, but also with its source, temperature, and concentration of iodine. It is now well established that glycogen possesses a higher degree of ramification as well as a much higher molecular weight than amylopectin. Various molecular weight determinations have been carried out on glycogen from different sources. Bridgman (118) using sedimentation rates, found values varying from 3.9 to 13.9×10^6 for a liver glycogen which was extracted in different ways. Meyer & Jeanloz (119) arrived at a minimum value of 6×10^6 for the water-extractable glycogen from mussels, using the osmometric method, whereas Chargaff & Moore (120) found 13.2×10^6 by sedimentation for the sedimentable glycogen from avian tubercle bacilli. Husemann & Ruska (121) measured the particle size of the *p*-iodobenzoyl derivative under the electron microscope and arrived at the value 6×10^6 .

The molecular weight varies considerably and in aqueous solution the phenomenon of micellar aggregation is superimposed. Thus it is possible to separate the solution-suspension of glycogen into a soluble and an insoluble fraction. Loring & Pierce (122) showed that by ultracentrifugation 78 per cent of rabbit liver glycogen and 80 per cent of the glycogen extracted from *Macrosiphum pisi* and *Aphis brassicae* could be separated. This glycogen is very pure and contains practically no nitrogen or phosphorus. Similar values were obtained for glycogen from the mussel *Anodonta*, i.e., 70 per cent insoluble fraction (119), and for glycogen from bakers' yeast (73 per cent) by electrodialysis (123). The viscosity of the insoluble mussel glycogen is twice that of the soluble fraction, whereas its degradation by β -amylase is only 30 per cent. By pro-

longed centrifugation of the aqueous suspension it can also be entirely separated. Bacterial glycogen could also be separated into two fractions by sedimentation (120).

Glycogen is less broken down by β -amylase than amylopectin, which is attacked to the extent of 50 to 55 per cent. This indicates a higher degree of ramification in glycogen. Thus, yeast glycogen suffers a 47 per cent degradation (123), commercial liver glycogen 46 per cent, mussel glycogen 30 to 40 per cent, depending on the fractions (119), and glycogen from *Zea mays* only 20 per cent (124). It is interesting to compare these values with the 47 per cent found by Meyer & Fuld (125) for the starch from glutinous rice. This starch, which is very soluble in cold water, has a chain length of twenty units, and gives a red color with iodine. Only its high viscosity and low molecular weight prevent its classification among the glycogens. The degree of ramification of a polysaccharide of the starch and glycogen types may be qualitatively evaluated by its coloration with iodine (126).

In view of viscosity, sedimentation and diffusion measurements (118, 119, 120) the glycogen molecule can no longer be regarded as spherical. From diffusion data it would seem that the shape is rather that of a flat ellipsoid with the ratio of the axes varying from one eleventh to one eighteenth.

The concept of glycogen being bound to protein and thereby becoming less extractable led Willstätter & Rohdewald (127) to assume the existence of a symplex which they called desmoglycogen. Ergle (128) found that not all the glycogen from the sclerotia of *Phymatotrichum omnivorum* could be extracted by hot water. However, Meyer & Jeanloz (119) proved that mussel glycogen is not chemically bound to protein. Their data indicate that the insoluble glycogen of high molecular weight cannot be entirely extracted by hot water because of mechanical inclusion in coagulated protein. Removal of protein without hydrolysis renders this glycogen extractable. This fraction would correspond to the desmoglycogen of Willstätter & Rohdewald.

Lichenin and isolichenin.—Acetolysis of the lichenin from Iceland moss, *Cetraria islandica*, gives octaacetyl-cellobiose, and hydrolysis of the methylated polysaccharide affords 2,3,6-trimethyl-glucose. Absence of ramification is deduced from the very small amount (approximately 0.8 per cent) of tetramethyl-glucose obtained (129). Meyer & Gürtler (130) showed that 1-3 links occur

to the extent of 27 per cent, beside the β 1-4 links. Upon hydrolysis of the methylated lichenin, 2,4,6-trimethyl-glucose was isolated as the anilide. The proportion of 1-3 links was determined by periodate oxidation of the polysaccharide, and verified by chromatographic separation of 2,4,6-trimethyl-glucose from the mixture resulting from the hydrolysis of fully methylated lichenin (131). The method of separation was worked out by Boissonnas (132). The presence of 1-3 links accounts for the differences between lichenin and cellulose, i.e., amorphous structure and solubility in alkali. Beside lichenin, and differing from it with respect to solubility in cold water, isolichenin could be isolated and fractionated into five polysaccharides (133).

Mesquite gum.—White (134) studied the hydrolysis of this gum and found that by partial hydrolysis 90 per cent of the arabinose, which is present in the furanoid form, is split off, whereas only 10 percent of the remaining molecule is attacked. The residual polysaccharide is still highly polymerized and may be separated by dialysis. Methylation and subsequent methanolysis afforded methyl trimethyl-D-galactoside, corresponding to the 2,4-dimethyl-galactoside isolated from the original gum after methylation and hydrolysis. The methyl trimethyl-galactoside consisted of the 2,3,4-derivative, 63 per cent, and the 2,3,6-derivative, 37 per cent. From this and previous data it follows that in all probability the araban portion of the gum is attached to galactose at the 3-position. Thus, the galactopyranose units would be bound to each other by 1-3 or 1-6 links, and every second galactose would carry a chain of four arabinofuranose units in the 3-position. The position of the methoxy-glucuronic acid remains to be established.

Flaxseed mucilage.—Anderson & Lowe (135) determined the content of D-galacturonic acid, L-rhamnose, L-galactose and D-xylose in this mucilage. The constituents occur in equimolecular proportions. Hydrolysis liberates xylose first, then L-galactose. An aldotronic acid containing D-galacturonic acid, L-rhamnose and L-galactose could be isolated. The physical properties of the mucilage would seem to indicate a branched structure.

Carrageenin.—This polysaccharide, isolated from Irish moss (*Chondrus crispus*) contains 31 to 34 per cent galactose, linked by 1-3 bonds, and a sulfate group in the 4-position (136). The other constituents are 1 to 2 per cent of pentoses, 20 per cent of ketoses and 20 per cent ash. Young & Rice (137) isolated 2-keto-D-glu-

conic acid (3 to 4 per cent) and demonstrated the presence of approximately 20 per cent of fructose, as determined by the resorcinol test. Hydrolysis of the polysaccharide was performed with oxalic acid in nitrogen atmosphere. In the presence of air, 2-ketogluconic acid is decomposed by hydrochloric acid to arabinose and arabonic acid. This explains the presence of the pentoses found previously. It is the first instance in which a 2-keto-onic acid is found in a polysaccharide.

Beef lung galactogen.—This galactogen differs from that of the snail *Helix pomatia*, and was isolated by Wolfrom *et al.* (138) by alcohol precipitation from the mother liquors resulting from a heparin preparation. It differs also in its rotatory power from the galactosan of marine algae, and from galactocarolose. No sugar other than galactose could be demonstrated after hydrolysis and chromatography.

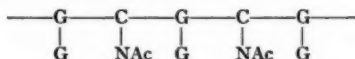
MUCOPOLYSACCHARIDES

Heparin.—This substance has been the object of numerous investigations, conducted by Wolfrom *et al.* (139) on its crystalline barium salt. The chemical composition of the crystalline heparin salts obtained from such various sources as beef lung, and dog, beef or pork liver, is essentially the same, and their biological activity is the same according to Wolfrom (140) and Risser (141) but varies from one to ten in the experience of Jaques, Waters & Charles (142).

According to Wolfrom, all the sulfur is present in sulfate groups which can be neutralized by barium, and the acidity of the salts is thus due to unlactonized carboxyl groups. The nitrogen is not present as a free amino or as an acetamido group, because it cannot be demonstrated as such. This, as well as the observation that periodate oxidation does occur to a small extent, is in contradiction to the findings of Charles & Todd (143). The uronic acid component was identified as D-glucuronic acid, and the amino sugar as D-glucosamine. Although the total number of identified constituents of heparin remains below that of chondroitin and mucoitin sulfate, it has not been possible to isolate other components. The significance of these investigations lies in the negative evidence relating to the amino group. This part of the molecule is of importance, since liberation of half of the amino groups, by breaking the bond in which they are involved, inactivates heparin. Wolfrom postu-

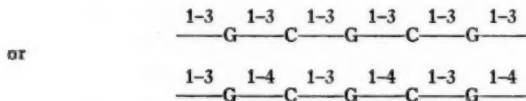
lates a bond of the anilide type and presents two types of formulae, which, however, fail to account for the properties of heparin as a polymer, and which have been severely criticized by Stacey (144). Dische & Meyer (145) demonstrated colorimetrically the presence of galacturonic acid in a crystalline barium heparinate prepared according to Charles & Scott (146).

Chondroitin sulfate.—Stacey *et al.* (147) have degraded this polysaccharide to a trisaccharide and eliminated the sulfate groups. After methylation and methanolysis, they isolated one third as methyl 2,3,4-trimethyl- α -D-glucuronoside and one third as methyl N-acetyl-3,4,6-trimethyl-chondrosaminide; the remainder consisted of methyl dimethyl-glucuronoside and methyl N-acetyl-dimethyl-chondrosaminide. From this it follows by calculation that in the original polysaccharide one half of the glucuronic acid is bound glycosidically as a terminal group, which implies a highly branched molecule of the following structure:



G=D-glucuronic acid; C=chondrosamine; Ac=acetyl

Meyer & Odier (148) came to a different conclusion. Their viscosity data point to an unbranched structure having a molecular weight of ten to fifty thousand. Measuring the latter by oxidation of the terminal group by iodine, they obtained a value of 31,600. Periodate oxidation reveals that, on the basis of this molecular weight, only the ends of the chain are attacked. In case of ramification, much more oxidant should be consumed than actually is utilized. The authors propose the following formulae:



G=D-glucuronic acid; C=N-acetylchondrosamine.

The sulfate group is attached in the 6-position to chondrosamine, as determined by periodate oxidation after removal of the sulfate groups.

Mucoitin sulfate.—Oxidative hydrolysis of this polysaccharide enabled Wolfrom & Rice (149) to isolate D-glucosaccharic acid,

indicating that D-glucuronic acid is the uronic acid constituent of mucoitin sulfate.

METHODS

L-Arabinose.—A convenient method for the preparation of L-arabinose from mesquite gum was reported by White (150).

Synthesis of aldoses.—The reductive desulfurization of thioesters has been applied by Wolfrom & Karabinos (151) to the preparation of aldoses. Ethyl thiol-D-ribonate tetraacetate, when refluxed with a fifteenfold quantity of Raney nickel in 80 per cent ethanol gave tetraacetyl aldehydo-D-ribose in 22 per cent yield.

The nitromethane method of Sowden & Fischer (39) has proved of value in the preparation of L-glucose and L-mannose (152) since comparatively large amounts of these sugars, and especially of L-glucose, may be obtained by this method more efficiently than by the cyanohydrin synthesis.

A reinvestigation of the sodium amalgam reduction of aldolactones and methyl aldonates to aldoses confirmed that the pH of the reaction mixture is the most important single factor influencing the yield of aldose. A pH range of 3 to 3.5 and the use of 2.5 to 3 atoms of sodium per mole of lactone at a temperature of 15° are recommended (153). By this method, D-arabinose was obtained in 57 per cent yield from the lactone or the ester.

Aldonolactones.—Pure aldonolactones may be recovered in good yield from the recrystallized hydrazides of the corresponding aldonic acids on treatment with nitrous acid (154).

Osazones.—The constants for the pure osazones of D- and L-arabinose have been redetermined by Hudson *et al.* The melting points were found to be about ten degrees higher than those previously recorded. Methyl cellosolve is recommended as the solvent in the preparation of the osazones (155).

Osotriazoles.—The phenyl osotriazoles corresponding to the four pentoses and the eight hexoses have now been described. In addition, the phenylosotriazoles of D-altro-, D-manno-, D-glucose, and D-galaheptose as well as those of L-rhamnose, L-fucose, lactose, cellobiose, melibiose and turanose (155, 156), and D-quinovose (157) were also prepared.

Analytical procedures.—The technique of chromatographic analysis has found wide application in carbohydrate chemistry, and the behavior on the column of a great variety of sugar derivatives, and, lately, of free sugars and sugar alcohols has been stud-

ied (97, 158). The occurrence of free fructose and glucose in cane juice was demonstrated by lyophilizing the juice, acetylation of the residue and chromatography.

Partridge (160) introduced the paper partition chromatogram, originally developed by Martin and co-workers (159) for the analysis of protein hydrolysates, as a tool for the qualitative analysis of mixtures of reducing sugars. Quantitative application of this method promises very interesting results, since it is possible to analyze less than one milligram of a sugar mixture, as was established by Flodd, Hirst & Jones (161). These authors studied several artificial mixtures as well as the hydrolysate from Cholla gum (*Opuntia fulgida*) which contains L-arabinose (6 parts), D-xylose (2 parts), D-galactose (2 parts), and uronic acid (1 part). Mixtures of methylated sugars can also be separated by this technique.

The separation of methylated sugars was also investigated by Hultin & Nordström (162) who used the method that permitted Tiselius & Hahn (163) to separate the products resulting from the enzymatic degradation of starch. Boissonnas (132) reduced his methylated glucoses to the corresponding glucitols, which were esterified with azobenzene-4-carboxylic acid and chromatographed on alumina. In this way several trimethyl glucoses have been separated. The determination of 2,3-dimethyl-glucose in the presence of 2,3,6-trimethyl- and 2,3,4,6-tetramethyl-glucose has also been realized by periodate oxidation (104).

Hurd & Zelinsky (164) separated several reducing hexoses and disaccharides by chromatography of their *p*-phenylazophenyl-polyacetylglycosides.

By using 2,4-dinitrophenylhydrazine, Neuberg & Strauss (165) succeeded for the first time in preparing osazones in quantitative yield.

Militzer (166) made use of the Kiliani reaction for the quantitative determination of aldoses and ketoses. Excess cyanide is measured by the Liebig-Dénigès method. Aldehyde, ketone, hemiacetal and hemiketal forms are estimated by this method, and hexoses are easily differentiated from pentoses, monosaccharides from disaccharides. In combination with the Willstätter-Schudel method, ketoses may be differentiated from aldoses.

Colorimetric methods have been established for the determination of pentoses in the presence of large amounts of glucose (167) and for the differentiation of glucose, galactose and mannose (168).

Specific color reactions for pentoses, methylpentoses, aldohexoses, ketohexoses (169) and uronic acids (169, 170) have also been reported.

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THE CHEMISTRY OF THE IMMUNOPOLY-SACCHARIDES

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Inasmuch as this is the first review of the immunopolysaccharides in this series, the aim of the reviewers has been the presentation of a relatively composite account outlining the state of our knowledge in the field as it stands today so that numerous and often noteworthy observations regarding some individual polysaccharides must of necessity be omitted. It is particularly appropriate that the immunopolysaccharides should be reviewed at this time for during the past two years we have seen most striking advances in the field, which have mainly arisen from the brilliant systematic work of American immunochemists. Notably there have been the methods outlined below which have been developed by Heidelberger and his associates (1) for the prevention of certain types of pneumonia by means of an immunotherapeutic procedure using various specific pneumococcal polysaccharides as vaccines. Also outstanding in regard to future work on polysaccharide synthesis is the discovery of the transformation of pneumococcal types (2) by means of a chemical entity composed essentially of desoxyribonucleic acid.

PNEUMOCOCCAL POLYSACCHARIDES

In 1923, Avery & Heidelberger (3) made the striking discovery that polysaccharides isolated from the mucinous capsular substances of various types of pneumococci were responsible for the serological specificity of each pneumococcal type. This and later work on the subsequent development of the discovery abolished the older view that proteins were the only significant immunizing antigens and introduced a new biological concept, namely that of the powerful determinative influence of carbohydrate residues in the immunogenic sense. It is of interest to note that in many bacteria the function of some of the proteins has now been relegated to the position of a vehicle for carrying the carbohydrate structure which determines the immunological type of the cell antigens. In the past two decades a vast amount of work has been done on the

serological properties and chemical structure of many bacterial polysaccharides. In the pneumococcus group it has been clearly shown that the forty or more types of pneumococci owe their type specificity to certain remarkable structural differences in their capsular polysaccharides. The latter, originally termed "soluble specific substances" (3), are best isolated from autolysed broth cultures of the organisms and purified by the standard methods of carbohydrate chemistry which avoid the use of heat, acids, and alkalis. The polysaccharides of Types I, II, and III were first examined by Heidelberger and his associates (4) who quickly found the marked chemical differences between them. The Type III material for example, was essentially free from any nitrogen-containing component and was the soluble salt of a strong acid, whereas the Type I polysaccharide was a weaker acid and contained a high proportion (5 per cent) of nitrogen. The specific optical rotations of the various polysaccharides differed markedly.

In general the pneumococcal polysaccharides fall broadly into two groups (5): the essentially nitrogen-free aldobiuronic acid-containing group as typified by Types II, III, VIII etc., and the amino sugar-containing group as exemplified by Types I, IV, XIV, etc. From time to time adequate reviews of the literature of the pneumococcal polysaccharides have appeared [e.g., Heidelberger (6), White (7), and Stacey (8)].

In the group of capsular polysaccharides the most striking development regarding structure has been the relating of serological cross reactions to the presence in the polysaccharide molecules of aldobiuronic acid building units, the presence of which also accounts for their power to give cross precipitin reactions with apparently unrelated polysaccharides such as gum acacia, cherry gum, *Rhizobium radiculicola*, *Azotobacter*, oxidised cellulose and types of Friedlander's bacillus, etc. (8). Reeves & Goebel (9) using methylation technique have shown that the pneumococcus Type III polysaccharide consists of a long chain of cellobiuronic acid units mutually joined through the 1 and 3 positions.

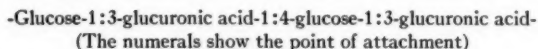


FIG. 1

This structure should be compared with those of the *Rhizobium radiculicola* polysaccharide (Fig. 2) and oxidised cellulose (Fig. 3),

-Glucose-1:4-glucose-1:4-glucuronic acid-

FIG. 2

FIG. 2

-Glucose-1:4-glucuronic acid 1:4-glucose-1:4-glucuronic acid-
FIG. 3

The powerful determinant property of the cellobiuronic acid unit was revealed by Goebel (13) who coupled the Type III cellobiuronic acid with an azoprotein and with the complex succeeded in producing a specific antiserum which conferred passive protection to mice against virulent Types II, III, and VIII pneumococci.

Until quite recently it had always been found that the specific polysaccharides, particularly in the pneumococcus group, failed to stimulate antibody production and so were classed as haptenes. Frequently, however, their serological activity was very powerful and they were shown to act as antigens in the white mouse, doses as low as 0.0001 mg. protecting such animals against one thousand fatal doses of virulent pneumococci (14). The subject of antipneumococcal immunization has recently been reviewed by Heffron (15) and now a new aspect on the subject is forthcoming from the important work of Heidelberger and his colleagues (16). The authors review early attempts to immunize humans by means of specific polysaccharides and then describe work which shows that the polysaccharides prepared by gentle methods [Heidelberger, Kendall & Scherp (17)] have a great potential value in the prophylaxis of pneumococcal pneumonia. Using the valuable microanalytical quantitative precipitin technique it was shown that when injected into humans in minute doses the type-specific polysaccharides (Types I, II, V, and VIII) behaved as powerful antigens and stimulated considerable antibody production. Field trials on large groups

of randomly selected army trainees over a period of years showed that single subcutaneous injections were effective in preventing pneumonia due to these types. Moreover the immunity lasted for a minimum period of six months, while the incidence of pneumonia was reduced in the nonimmunized and the carrier rate also was significantly lowered. Extension of the work (18) has shown that immunized humans contained after two months sufficient amounts of type specific antipolysaccharide antibodies to account for the favourable results of the immunization procedure. This investigation also showed that the group-specific or "C" polysaccharide (19) played little or no part in the immunization process. This "C" polysaccharide can be isolated from the somatic part of the cell (20) in which it is closely associated with nucleic acid constituents. It contains nitrogen (5.0 per cent), amino sugar, galactose, and acetyl residues. It occurs in the cell in conjugated form since in combination with a fatty acid it constitutes the so-called heterophile antigen of the pneumococcus. An unusual antigenic relationship between the Type XIV polysaccharide and certain human A red cells may arise from close structural similarities (21). Injection of the Type XIV pneumococcus into the horse gives rise to an antiserum which agglutinates red cells of all the four main blood groups and gives a precipitin reaction with the Type XIV pneumococcus specific polysaccharide. Certain fatal reactions observed following the use of Type XIV pneumococcus antisera appeared to arise from this unusual cross reactivity with erythrocytes. It was significant that a blood group A polysaccharide could absorb out all the Type XIV anticarbohydrate antibodies. It is evident that the Type XIV specific polysaccharide and the blood group A factor, both of which contain among other constituents D-galactose and N-acetyl D-glucosamine units, possess some carbohydrate structures in common.

Apart from the detailed knowledge of the structure of the Type III polysaccharide, our information on the other polysaccharides is limited to scattered observations on their general chemical and physical properties and on their monosaccharide components [see listing by Boyd (22)]. Recently, however, some measurements have been made by Record & Stacey (23) on the physical properties of undegraded samples of Types I, II and III polysaccharides. These show molecular weights generally of the same order, 150,000 to 500,000, and with each specific polysaccha-

TABLE I
PNEUMOCOCCAL POLYSACCHARIDES SEDIMENTATION
AND DIFFUSION DATA

Type	$S_{20} \times 10^{13}$ ($c \rightarrow 0$)	$D_{20} \times 10^7$ ($c \rightarrow 0$)	Molecular weight	Fractional ratio
I	6.5	2.00	170,000	3.2
II	7.2	0.75	500,000	6.0
III	4.3	1.60	140,000	4.3

ride a high fractional ratio indicated a departure from linearity in the shape of the molecules.

Physical chemical studies on the Type I polysaccharide have been made by Heidelberger & Albery (24). Electrophoresis of a commercial preparation of the Type I specific polysaccharide was carried out at several pH values and there were separated out 15 per cent of nucleic acid and 10 per cent of the "C" polysaccharide which was electrically immobile. Only one component could be seen in the ultracentrifuge and the run at 50,000 r.p.m. was typical for very elongated molecules.

The knowledge that each one of the large number of pneumococcal types owes its specific type characters to the presence of a capsular polysaccharide of a particular molecular structure is of the utmost importance when viewed in the light of the new discoveries of Avery, MacLeod & McCarty (2) on the transformation of pneumococcal types. Griffith in 1928 (25) had succeeded by cultural methods in transforming an attenuated and nonencapsulated rough (R) variant into fully encapsulated and virulent smooth (S) variant of a heterogeneous specific type and this work was confirmed and extended by Dawson & Sia (26). Avery and his colleagues have now shown that a Type II, R, pneumococcus can be transformed into a Type III, S, pneumococcus by the agency of what was essentially a desoxyribonucleic acid of high molecular weight extracted from the Type III pneumococcus.

Not only is this discovery an authentic case of a specific mutation caused by a chemical entity but it also represents the isolation of the determinant part of the enzyme system responsible for specific polysaccharide synthesis. A speculation on the role of the specific polysaccharide as a pattern or "starter" in the type transformation has been made by Stacey (27). Other pneumococcal

types have now been transformed and it has been shown that the transformation in Types II, III, and VI can be inhibited by desoxyribonuclease (28). These discoveries, together with the work on polysaccharide synthesising enzymes such as starch and glycogen phosphorylases, levan and dextran sucrases, etc., constitute a remarkable advance in polysaccharide biochemistry.

POLYSACCHARIDES FROM PATHOGENIC STREPTOCOCCI

Few polysaccharides from human pathogenic streptococci have been examined chemically. The type-specific substances are essentially proteins (29). Perhaps the most striking streptococcal polysaccharide is the serologically inert viscous polysaccharide obtained from mucoid forms of Group A streptococci by Kendall, Heidelberger & Dawson (30). This substance appears to be closely related to hyaluronic acid of the animal body (31) and its significance in invading organisms is shown by the work of McClean (32).

IMMUNOLOGICAL PROPERTIES OF DEXTRANS

Serological relationships exist between certain pneumococcal type-specific polysaccharides and the polysaccharides known as dextran and levans and our knowledge on these is indicated below. Dextrans are polyglucoses produced from sucrose by a variety of organisms [Stacey (33), Evans & Hibbert (34)].

Chemically degraded dextrans are acquiring significance as substitutes for plasma (35). Certain dextrans (36) including those formed by cell-free filtrates of *Leuconostoc mesenteroides* (37) react markedly with antisera of pneumococcus Types II, XII, and XX and inasmuch as dextrans contain no amino sugar or uronic acid residues but are straight or branched chain polyglucoses containing a preponderance of 1:6 α -glucosidic linkages (38, 39), considerable work is needed to place this cross specific relationship on a structural basis. Since nitrogen-free degraded dextrans are not antigenic (40) it has been suggested that immunological relationships may be found in the prosthetic groups of the macromolecular structures (93).

Hehre, Neill and colleagues (41) have carried out extensive studies on the serological properties of dextrans and have found that antisera to the dextrans are of special significance in determining dextran impurities in cane and beet sugar.

With the further development of dextran derivatives as plasma

substitutes it is likely that serological reactions will be widely used for detection of dextran in tissues. An interesting branched chain dextran has recently been described by Stacey & Swift (42).

IMMUNOLOGICAL PROPERTIES OF THE BACTERIAL LEVANS

Levans are acid-labile fructofuranose-containing polysaccharides which are synthesised by plants and by a wide range of aerobic bacteria from sucrose or raffinose as substrates (34). The repeating unit in all the levans so far examined by the methylation technique appears to be identical and it consists of contiguous chains of about twelve fructofuranose units mutually linked through the 2 to 6 positions (43, 44). Wide differences exist in the physical properties of various levans and these may be due to varying degrees of aggregation of the repeating units.

The serological properties of bacterial levans are of great interest because certain levan-forming (as well as dextran-forming) organisms such as *Streptococcus salivarius*, *S. varidans*, etc., are pathogenic to man and in some way are concerned with bacterial endocarditis (45). Others are plant pathogens, e.g., *P. pruni* (44), *P. mors prunorum*, etc. The serological aspects have been the special subject of study by Hehre, Neill and their colleagues. In one investigation (46) the levans formed from sucrose or raffinose by a spore-forming bacillus ("N 9") of plant origin and by a strain of *S. salivarius* were closely related serologically. The important observation had previously been made (47) that the antisera could be prepared by injection of organisms which had been grown on sucrose or on raffinose, but no antibodies could be stimulated by organisms grown on glucose. It thus appeared that the serologically reactive levans could only arise from a sucrose substrate. They cross reacted surprisingly enough with strains of Type XX pneumococcus. Like the dextrans, levans can be synthesised from sucrose by cell-free enzymes (48). The important observation was made (49) that the levans synthesised by cell-free enzymes from two levan-formers (*S. salivarius* and a spore-forming bacillus "N 9") had serological properties similar to those described for the powerful serologically reactive levans synthesized by the living cells (50). A wide investigation of the problem was then undertaken by Hehre and his colleagues (46) who studied the cross reactions between levans from numerous organisms, and antisera which were known to react in high dilution with the levans previ-

ously investigated. All exhibited a high degree of serological similarity and it was concluded that all the levans studied possessed closely related serological properties and that serological procedures can safely be applied for detection of bacterial levans in laboratory culture fluids and in the various materials in which they occur in nature. This work supports an earlier suggestion by Cooper (51) that levan formation may be valuable in determinative bacteriology for identification of species.

An interesting line of investigation on the significance of *Pseudomonas mors prunorum* levans in the resistance of plum trees to bacterial canker has been opened up by the work of Erikson (52). This *P. mors prunorum* levan possesses the typical levan structure (53). The notable plant pathogen, *Phylomonas tumifaciens*, produced a polyglucose (54) having the glucose residues linked presumably through the 1 to 3 positions.

MENINGOCOCCAL POLYSACCHARIDES

The specific polysaccharide of Type I meningococcus has been prepared and characterised by Scherp & Rake (55) but was later shown to contain 20 per cent of a serologically active impurity (56). Following the lines of study previously explored in the pneumococcus group, Kabat and his colleagues (57) investigated the effectiveness of an electrophoretically pure Type I meningococcal polysaccharide as an antigen in human beings. The polysaccharide was remarkable for its high phosphorus content (5 to 6 per cent) and its reactivity with basic protamine-like protein. The polysaccharide gave a poor antigenic response in man but did form some precipitin and protective antibodies.

Carbohydrate-lipin complexes have been isolated from various meningococcal strains by Boor & Miller (58) by an extraction method using trichloroacetic acid at 0°C. The complex possessed toxic properties and the yields varied with each strain. Acid hydrolysis of the complex yielded a polysaccharide which reacted specifically with sera prepared both against the whole cells and against the carbohydrate-lipin complex.

A review has recently been written (59) on the vast number of polysaccharides, both serologically specific and inert, produced by *M. tuberculosis*. Polysaccharides have been isolated from whole and autolysed cells, from the waxes, from the somatic region, and from the metabolic fluids. The general properties of many of these have

$$A \xrightarrow{\begin{smallmatrix} \vdots \\ \text{G} \\ \vdots \end{smallmatrix}} \begin{smallmatrix} \text{M} \\ \vdots \end{smallmatrix} \left[- \text{G}^1 - \text{A}^1 - \right]_n \begin{smallmatrix} \text{A}^1 - \text{M}^{\text{M}-1} \text{A} \\ \vdots \\ \text{N} \\ \vdots \end{smallmatrix}$$
$$R^1 - {}^2A^1 - {}^2M^1 - \left[\begin{array}{c} R_1 \\ | \\ A_1 \\ | \\ {}^2M^1 - {}^2M^1 - \end{array} \right] - N \dots$$

From the immunizing point of view the most important complex polysaccharides are found among the heterophile antigens of gram negative bacteria such as those in the *Salmonella* group, e.g., *Bact. typhosum*, *Bact. shigae*, etc.

These were discovered simultaneously by Boivin & Mesrobianu (63) using trichloroacetic acid extraction and Raistrick & Topley (64) using tryptic digestion and appear to consist of polysaccharides combined with phospholipins. They are related to certain heat-stable tissue polysaccharides such as the Forsmann antigen (65) and Wasserman antigens (66).

The bacterial phospholipins constitute the "O" or somatic antigens which together with the less well defined Vi antigen (the Virulence antigen, probably a complex polysaccharide at the cell surface) appear to be those component parts of the vaccines which on injection give rise to the protective antibodies. They are toxic and when degraded into their building units, only the carbohydrate portion appears to have hapten properties, while the lipoprotein is toxic and relatively nonantigenic.

Various methods have been used to extract the O-antigens including the extraction of cells with reagents like trichloroacetic acid, trypsin, diethylene glycol, phenol, guanidine, etc., followed by precipitation of the antigens as amorphous water-soluble powders by alcohol or acetone. All methods appear to give closely related complexes of high molecular weight. The *Bact. typhimurium* (aertrycke) somatic antigen contains a complex constituted of four components: a specific polysaccharide, a polypeptide, an acetyl polysaccharide, and a phospholipin (67). The constituents produced on acetic acid hydrolysis have been examined in some detail (68, 69, 70). The complex contained 69 per cent of polysaccharide, 16 per cent of a conjugated protein, 3 to 4 per cent of lipin, and 8 per cent of an alcohol-soluble polysaccharide. The alcohol-insoluble polysaccharide, which readily combined with protein, was considered to be built up from units of D-glucose (19 per cent), D-mannose (21.5 per cent), and D-galactose (19 per cent). The *Bact. typhosum* antigen is a similar complex containing 50 to 60 per cent of a polysaccharide ($[\alpha]_D + 114^\circ$ in water), 16 per cent of an insoluble polypeptide, 10 to 20 per cent of a soluble nitrogenous constituent, and 3 to 4 per cent of a lipid component. The polysaccharide yielded D-galactose, D-mannose, and D-glucose on hydrolysis. It appears that the *Bact. typhosum* polysaccharide contains D-glucosamine also as a constituent unit.

Independent but closely parallel studies have been carried out on *Bact. shigae* mainly by Morgan and his colleagues (71). The somatic antigen of *Bact. shigae* has been prepared in an actively

antigenic form by the same isolation methods as those described for other of the *Salmonella* O-antigens, i.e., extraction with trichloroacetic acid or diethylene glycol. The purified products were typical of those of *Salmonella* O-antigens and have been shown to consist of toxic phospholipin polysaccharide-protein complexes. Morgan & Partridge (71) have been able to dissociate the phospholipin from the polysaccharide-protein complex and to demonstrate that the polysaccharide-protein complex alone was both antigenic and toxic. The polysaccharide-protein complex could be dissociated with 90 per cent phenol to give a nonantigenic haptene polysaccharide with a high viscosity. It could, moreover, be recombined with the protein portion in slightly alkaline solution to form a reconstituted "complete" antigen. The protein part was a conjugated protein very similar to that obtained from the O-antigens of other *Salmonella*. Morgan & Partridge showed that agar, gum acacia, and the blood-group A substance could be combined with it to produce immunizing antigens. Rough variants of *Bact. shigae* yielded negligible amounts of these complex antigens on extraction with diethylene glycol, but similar materials were obtained from other strains of dysentery bacilli (e.g., Flexner). As with many other immunopolysaccharides, the criticism may be made that the dysentery-antigens have not yet been characterised as to homogeneity in the Tiselius apparatus nor in the ultracentrifuge, nor have detailed quantitative precipitin-studies been carried out to determine the relation of the isolated antigenic materials to the intact cell. The role of phospholipin, polysaccharide, and conjugated protein in the reaction with antiserum also have not yet been studied quantitatively. Some quantitative precipitin studies were carried out by Morgan (72) on the amount of antibody precipitated by the dysentery polysaccharide, and by the polysaccharide-protein complex from antisera to the polysaccharide-protein complex. Both materials precipitated the same amount of antibody from the sera within the error in determining the point of maximal precipitation; about 40 per cent as much polysaccharide was needed for complete precipitation of antibody as was needed of the polysaccharide-protein complex. No detailed studies in polysaccharide structure have yet appeared though it is of interest to note that the nontoxic *Bact. shigae* O-hapten polysaccharide component yielded on hydrolysis D-galactose, L-rhamnose, and N-acetyl-D-glucosamine. The toxicity of these antigenic preparations appears

to be largely due to the integrity of undegraded complex. Examination of the individual components revealed many interesting facts. Thus with *Bact. shigae* the specific polysaccharide failed to induce any demonstrable antibodies, while the polypeptide component engendered homologous precipitins of low titre but no agglutinins against *Bact. shigae*. The "reconstituted" polysaccharide-polypeptide complex, however (made by mixing the two substances in formamide), induced the formation of specific immune serum of high titre. Goebel, Binkley & Perlman (73) have confirmed and extended some of Morgan's findings on *Bact. dysenteriae* (Shiga) (Type V). These workers studied the antibody response in human volunteers to injection of minute doses of the highly toxic complex polysaccharide and their results were quite promising from the prophylactic point of view. An interesting study was made by Treffers (74) on the effect of acetylating the somatic *Bact. shigae* polysaccharide complex. This treatment appeared to have a remarkable effect in decreasing the toxicity of the complex without appreciably affecting its protective power. Acetylation also caused significant detoxification with the *Bact. typhosum* O-antigen.

POLYSACCHARIDES OF *SALMONELLA MARCESCENS*

It has been shown that the culture filtrate of this organism contains a polysaccharide substance having interesting cytological properties. The filtrates obtained by growing the organisms on a synthetic medium induce a specific skin reaction which it is thought is related to the haemorrhages and regression which an injection of the polysaccharides causes in mouse tumours. Extensive studies by Shear and co-workers (75, 76, 77) showed that mice bearing chemically induced tumours died on injection with the *S. marcescens* polysaccharide, while an identical dose of the polysaccharide was without effect in control animals. The toxicity is ascribed to extensive degradation products due to the rapid breakdown of the tumour. A degree of tolerance can be established in cancerous mice by successive injection with small amounts of the polysaccharide. It was shown that after twenty-four hours, 0.1 mg. injected intraperitoneally into cancerous mice caused extensive microscopic necrosis of the tumours, and that various types of tumours were affected in this manner. Further cytological studies by Deller & Shear (78) showed that the nuclei of tumour cells underwent degenerative changes, with a "blistering" of the cell mem-

brane. Normal rapidly dividing cells were similarly damaged by the polysaccharide. Further studies in this field are awaited with interest.

RELATIONSHIPS OF CERTAIN IMMUNOPOLYSACCHARIDES TO VIRUSES

It has been known for some time that red blood corpuscles are agglutinated by some viruses and this phenomenon has been used as a tool for measuring the amount of virus present in various specimens, and in the study of problems of immunity against influenza (79, 80). Based on a similarity of the haemagglutination by virus to that by enzymes of *V. cholerae*, it was concluded by Burnet and his colleagues (81) that viruses exert their effect by similar enzymes or enzymatically active groups on the surface of the virus particle. This led to ideas for the study of the role of enzymes involved in virus invasion and for possibilities of a chemotherapeutic defense against them.

It was then found that virus haemagglutination could be inhibited by such substances as normal sera, extract of animal tissues, allantoic fluid, human tears, etc. The inhibitor appeared to be quite distinct from the A, B and Rh blood group substances. Burnet *et al.* (82) found that glandular mucin in dilute solution possessed all the essential properties of the inhibitor and also that the blood group O mucopolysaccharide is active in a dilution of 1:1,200,000 and the blood group A mucopolysaccharide in a dilution of 1:200,000. The activity of both is destroyed by the *V. cholerae* enzyme. It appeared that the substrate for the virus enzyme is a mucopolysaccharide closely related to those present in red cells responsible for A, B or O group specificity.

Work in the virus field was then brought directly into the field of immunopolysaccharides. Green & Woolley (83) tested a number of simple and complex carbohydrates for their power to inhibit the agglutination of chicken erythrocytes by influenza A virus. Apple pectin, citrus pectin, flaxseed mucilage, blood group A substance, gum acacia, gum myrrh, and an extract of chicken erythrocytes were active. Apple pectin appeared to alter both the corpuscles and the virus. When introduced into the allantoic cavity of embryonated eggs half an hour before injection of the virus, 25 to 50 mg. prevented multiplication of relatively large amounts of the virus in the great majority of eggs, while in some instances it

was effective against much larger doses. It was rather less active when given one hour after the virus. Of other carbohydrates tested, gum acacia alone showed a significant inhibitory effect in the embryo, and then only when injected before the virus.

Horsfall & McCarty (84) investigated the effect of a superimposed intranasal infection with a nonhaemolytic streptococcus upon the lesions produced by the pneumonia virus of mice. The combined infection resulted in a significantly less severe pneumonia than in mice receiving the virus alone. Several other organisms exerted a pronounced modifying effect on virus pneumonia and these and other observations eventually led to the finding that the effect was due to polysaccharide material. Among various polysaccharides which gave a striking effect were the somatic polysaccharides of *Bact. shigae*, the blood group A polysaccharide, a streptococcus capsular polysaccharide, etc. That the effect was relatively specific was shown by the fact that the pneumococcal polysaccharides Types I and II, the pneumococcus C substance, gum acacia, heparin, and an aldobiuronic acid from Friedlander's bacillus failed to modify the course of the disease. The mechanism of the chemotherapeutic activity of the polysaccharides is not yet clear but evidently a new aspect on virus studies has been opened up.

POLYSACCHARIDE COMPONENTS OF ALLERGENS

A monumental piece of work is steadily being built up by Spies, Coulson, Stevens and their colleagues (85, 86) on the allergens, particularly those from cottonseed. The main investigations have been on the immunological side but now the chemical nature of the determinant groups responsible for hypersensitivity of the allergenic type is being revealed.

From cottonseed all the allergenic fractions appear to be contained in a protein-polysaccharide fraction (87), associated with cottonseed globulin, which was purified by a wide variety of fractionation procedures. The predominant antigen was a peptide-like substance but there was strong evidence that complex polysaccharides of the mucoprotein type play an immunological role. Ragweed pollen, kapok seed, castor beans, etc., contain related antigenic mucoproteins.

BLOOD GROUP SPECIFIC POLYSACCHARIDES

There is a rapidly growing literature on the important blood group specific carbohydrates and since these have been reported on quite recently (88, 89), the reviewers simply call attention to them here. The only structural studies so far reported are those of Bray, Henry & Stacey (90).

Until 1945 it was thought that these substances were serologically active solely in respect to their power of combining with antibody and being able to inhibit in extremely high dilution iso-haemagglutination of red cells. Now it has been found (91) that they are capable of behaving as antigens in man and of producing sera of types suitable for blood grouping. This discovery has opened up new prospects in the field; it has revealed (92) that many older preparations were impure and that one must have reliable biological methods for detection of purity in polysaccharides having closely related chemical and physical properties. There is undoubtedly an urgent need for new criteria of purity in the whole of the polysaccharide group, while there is even greater need for clear cut quantitative methods for estimating polysaccharides when they are firmly bound to other groups.

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X-RAY CRYSTALLOGRAPHIC STUDIES OF COM- POUNDS OF BIOCHEMICAL INTEREST

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The years 1944-1947, which this review should cover, are years exceptionally rich in published papers on x-ray crystallographic analysis. Partly this is the effect of the war years; much of the work which appeared in print in this period was actually carried out earlier and publication delayed. But partly, it is the effect of the growing power of x-ray analytical methods which has been noticed in previous years.

It is interesting to read in Sponsler & Dore's review on this subject (1936), the first of the *Annual Review* series,

For such crystalline substances as the sugars and amino acids, complete knowledge of the crystal structure would show the arrangement of the atoms within molecule as well as the arrangement of the molecules within the crystal;

and to realise that no such knowledge was available twelve years ago, at the time of this first review. In the interval, the first detailed analyses of compounds of both these types have been carried out and have been reported, by ones and twos, in the last three reviews. But the widespread application of new methods of x-ray analysis, foreseen by Huggins in 1939, was delayed by the war, as reported by Fankuchen in 1941.

Now we have, crowded into one short period, results in a wide variety of fields. The analyses of geranylamine hydrochloride and *d*- α -bromo-, chloro- and cyano-camphor are first examples in the terpene series. Two more amino acid structures, nickel glycine and copper *d*- α -amino-butyrate have been partly solved, and we have also a preliminary account of two pyrimidines. The crystal structures of sucrose sodium bromide complex among the sugars and of cholesteryl iodide among the sterols have been found. The chemical structure of penicillin, in the form of three benzylpenicillin salts, has been, in detail, solved by x-ray methods. In addition to this series of structure analyses on compounds of characteristically "biochemical" types, we have a background series of analyses on more purely organic compounds. These also should have a certain interest for the biochemist, both as illustrations of the present state of x-ray analysis, and of particular features of chemical

and crystal structure which may appear later in more strictly biochemical systems.

That there is now this wider experience of structure analysis available seems to make it desirable to treat the subject matter of the present review rather differently than in previous reviews. It is possible now to give actual examples of the arrangements of atoms within molecules and molecules within crystals in all the different biochemical fields mentioned. These arrangements are in type much as predicted from general considerations described earlier; it is their detailed and individual character that makes them particularly interesting. But in practice, it is difficult to divorce any account of detailed molecular and crystal structures from some account of the methods of x-ray analysis used in their derivation, since on these depend the accuracy of the actual structure determination. I propose therefore, in this review, to link the account of the different structures together in a general account of the present methods of detailed x-ray analysis. This will be a natural continuation of the report made last year by Dr. Fankuchen on the scope of x-ray methods in general in the biochemical field.

This account of detailed x-ray structure analyses should indicate both what has been and can be achieved by crystallographic methods, and also what cannot so far be discovered. As the molecules become larger, or the crystal structures less regular, the accuracy and scope of the knowledge gained by x-ray methods necessarily decreases. In the examination of high polymers, and particularly of proteins and viruses, by far the most striking advances in our knowledge of crystal structures have come recently from electron microscopy and not from x-ray diffraction. But the information obtained by these two methods is closely related, and combined it forms a natural conclusion to the survey presented in the first part of this review.

RECENT X-RAY ANALYSES OF THE STRUCTURE OF ORGANIC MOLECULES

The ease with which any crystal structure may be analysed and the amount of x-ray diffraction data necessary for the process depend upon the complexity of the actual structure under examination. Thus for a crystal, such as diamond, with high symmetry and few atoms in the crystal unit cell, observations and calculations on the intensities of as few as nine x-ray reflections (1) are

sufficient to discover the arrangement of the atoms in the crystal. The process of x-ray analysis used in this case is a simple one of testing out a limited number of suggested atomic arrangements. For each trial structure, it is possible to calculate two characteristic quantities, the structure amplitude F and phase constant α , for each observable x-ray reflection. The square of the structure amplitude or structure factor is proportional to the intensity of the observed x-ray reflection and so the calculated values may be checked against those observed. In general, the relation between x-ray intensities and atomic positions is a very sensitive one, so that good agreement between calculated and observed intensities is often sufficient by itself to establish a particular atomic arrangement as correct, certainly sufficient with a structure like diamond.

Again, in diamond, the atomic arrangement is such that the actual distances between the atoms are known, once the unit cell dimensions are known. This is because, in this highly symmetrical crystal, the parameters which define the positions of the atoms in the unit cell bear a special relationship to the cell length. As a consequence, the carbon to carbon distance in diamond can be measured very accurately. Mrs. Lonsdale finds that it varies a little in different diamonds, from 1.54465 Å to 1.54440 Å (2), figures which agree well with the mean value given by Riley, 1.54453 Å (3). But this kind of accuracy of bond-length determination cannot be reached in any other structure mentioned in this review. In all of these, the atomic parameters bear complex relations to the cell lengths and can be found with very variable degrees of precision.

In addition to the discovery of the arrangement of the atoms in space and of the distances between them, it is of general theoretical interest to find the actual electron density throughout the crystal and particularly over the atoms and the chemical bonds which join them. The electron density, ρ , may be found at any point xyz within the crystal by the calculation of Fourier series of the form

$$\rho_{xyz} = \sum_{h=-\infty}^{\infty} \sum_{k=-\infty}^{\infty} \sum_{l=-\infty}^{\infty} |F_{hkl}| [\cos 2\pi(hx + ky + lz) - \alpha_{hkl}].$$

This may appear a formidable equation, and indeed it is formidable to apply to complex structures; in many cases, however, it simplifies considerably, and particularly for certain forms of calculation such as that of the electron density projected on a plane or along a

line. For any calculation of electron density, F values derived from the observed intensities are used but the phase constants α are usually derived from a trial structure. As such, they may not at first all be correct and the calculation then has to be repeated, or refined, until the clearest possible evidence of the positions of the atoms in the structure is obtained. The first electron density calculations on diamond were carried out as early as 1925 (4) and have been repeated since (5). They are theoretically interesting since they show finite electron densities along the covalent bonds in diamond.

The chemical structure of diamond in the ordinary sense, the knowledge of how the atoms are linked together within the molecule, followed from the first calculations on the intensities of the x-ray spectra. Carbon atoms bonded together were obviously those close to one another. And in general, in organic crystal structures, it is found that atoms joined by covalent bonds are within 1 to 2 Å of one another, depending on the nature of the atoms involved, while longer distances imply the existence of weaker forces of attraction. Relatively few structures of organic molecules have, in fact, been solved in this way, though there are examples; hexamethylene tetramine (6) is one and now penicillin is another. It is more usual to find in crystal analyses that the molecular structure is taken roughly as given. This limits the number of possibilities which have to be investigated in seeking for a solution of the crystal structure and does not, in any way, detract from the value of the solution when found.

There are a few organic compounds in which observations and calculations on x-ray intensities alone can fix, with reasonable accuracy, the atomic positions. One nice example is the structure of adamantane (Fig. 1) which is very like that of diamond and has a similar carbon to carbon distance, $1.54 \pm .016$ Å [Nowacki (7)]. The structure of this molecule is very like that of hexamethylene tetramine, but the two crystal structures differ in actual molecular packing. The intermolecular distances in hexamethylene tetramine are slightly smaller than those in adamantane, 3.9 Å as against 4.18 Å, between nearest neighbours, which suggests increased forces, of attraction between nitrogen and methylene groups compared with those in the purely hydrocarbon system of adamantane. Recent more exact calculations on hexamethylene tetramine [Shaffer (8)] indicate the existence of rotational thermal vibrations in the crystal and one would expect the same for adamantane. It

is interesting to compare with these two the crystal structure of quinuclidine (I) [Nowacki (9)]. This is also cubic but statistically

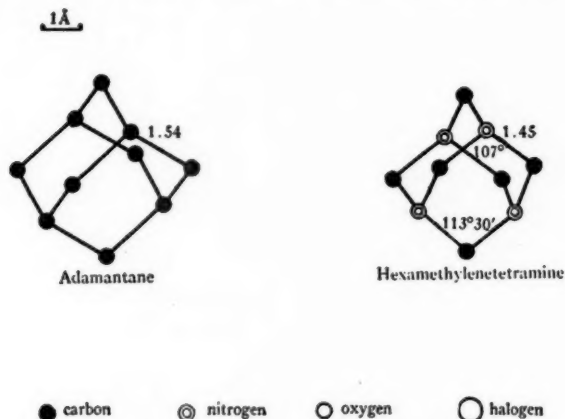
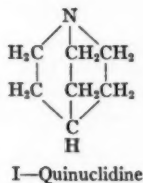


FIG. 1.—Molecular dimensions of adamantane and hexamethylene tetramine. Here and in the following diagrams the atoms are represented as above.

The hydrogen atoms are omitted. Interatomic distances are recorded in Angström units.

disordered, probably due to the onset of more violent rotational movements. As a result the atomic positions have not been determined.



As the crystal structures become more complex the calculation of Fourier series is necessary, not only to show the variation of electron density in the different types of system but actually to find, as exactly as possible, the atomic centres. In a number of crystal structures the calculation of the electron density in projection is sufficient to fix the atomic positions. The aromatic hydrocarbon, coronene, for example, lies in such a way in the crystal

unit cell that all its atoms project, clearly separate from one another, on one crystal plane (Fig. 2). The interatomic distances can

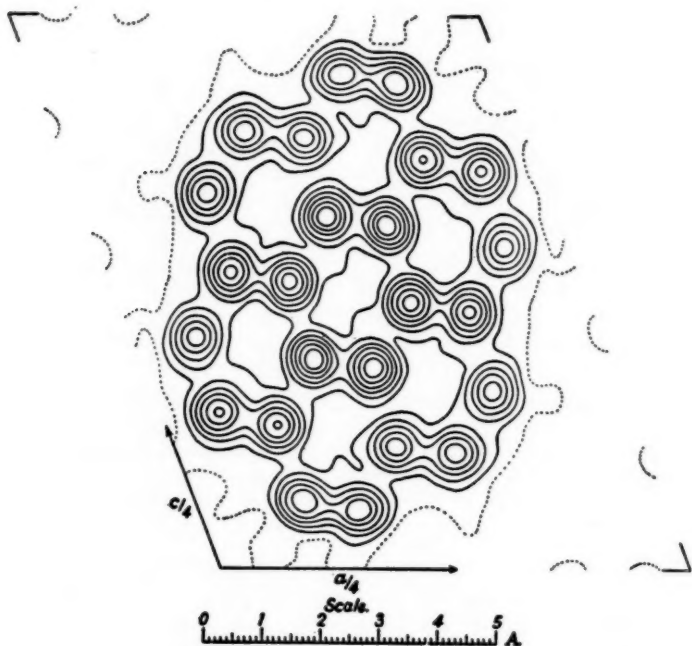


FIG. 2.—Projection of electron density along the b axis in coronene. The plane of the molecule is inclined at about 44° to the projection plane. Each contour line joins points of equal electron density, the electron density increment being approximately 1 electron per \AA^2 , with the 1 electron line dotted. [Fig. from *J. Chem. Soc.*, 609 (1945)].

be fixed within $\pm 0.02 \text{ \AA}$, and show interesting variations among themselves [Robertson & White (10)]. These variations correspond roughly with what might be expected on theoretical grounds. The usual sum of possible resonance forms suggests that the bonds of type a (Fig. 3) should have 67 per cent double bond character and those of type b , 33 per cent. However, no differentiation would be expected theoretically between bonds of type b and c which appear different in the electron density patterns.

There are a number of other structures in which electron density projections give good evidence on the molecular structure. Interesting examples are biphenylene (11) and 1,2,3,4-tetraphenylcyclobutane (12), both of which contain four-membered rings. Examination of the latter compound was actually undertaken in connection with certain chemotherapeutic researches, in

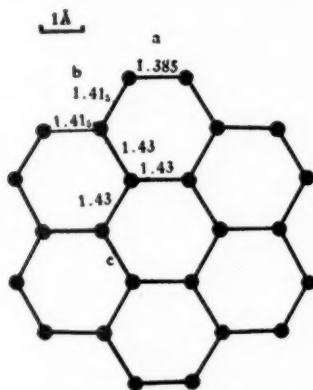


FIG. 3.—Dimensions of the coronene molecule.

order to settle the constitution of toxic irradiation products of the drug stilbamidine. Dimerisation was suspected, and the irradiation products were isolated and converted into the hydrocarbon mentioned above, together with small quantities of what is probably a lower melting isomer. These were submitted for x-ray analysis, which has so far established the structure of the high melting product. This molecule proves to be the centrosymmetrical isomer of 1,2,3,4-tetraphenylcyclobutane and the molecular symmetry has interesting consequences. The stereochemical configurations of the benzene rings about the two bonds of the cyclobutane ring are different, *cis* and *trans* respectively, and this difference seems to be associated with changes in length of the carbon to carbon bonds. From the projections the lengths appear to be 1.63 and 1.49 Å but the three dimensional x-ray analysis now in progress indicates less extreme deviations from the normal.

There are, indeed, relatively few organic crystal structures from which exact measurements of bond lengths can be obtained with-

out the calculation of a part of the full triple Fourier series which will give the electron density, at least at certain points in three dimensions within the crystal cell. The calculations involved are far more laborious than those mentioned so far but they have already been carried out on several compounds. These include *p*-dinitrobenzene (13), pentaerythritol tetranitrate (14) and dibenzyl (15). Here the bond lengths are probably the most accurately measured so far—those in dibenzyl are given to $\pm .01 \text{ \AA}$ —and again we have

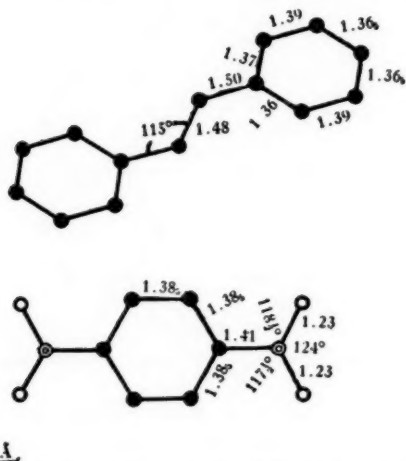


FIG. 4.—Dimensions of the molecules of dibenzyl and *p*-dinitrobenzene.

a picture of small variations in bond length throughout the molecule, variations beyond those that would be expected from the chemical formula as generally written and even from the formula as it might be expected to be modified by resonance. In dibenzyl, for example, there are small, but probably real, variations in the lengths of the bonds in the benzene ring itself and the central— CH_2CH_2 —bond is definitely shortened compared with the standard diamond carbon bond length (Fig. 4).

Another compound on which three-dimensional x-ray analysis has recently been carried out, the complex between quinol and sulphur dioxide, is particularly interesting, not because of the accurately measured bond lengths, but on account of the arrange-

ment of the molecules in the crystal [Palin & Powell (16)]. This is illustrated in Fig. 5. The quinol molecules are linked through hydrogen bonds to form framework structures extended in three dimensions throughout the crystal. Two such frameworks, independent of one another but inseparable, interpenetrate and form

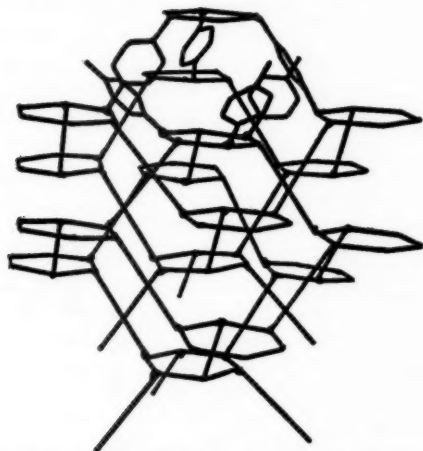


FIG. 5.—The structure of quinol. The model shown illustrates the interpenetration of two similar hydrogen-bonded cageworks. The large hexagons are the hydrogen bonds. The smaller hexagons in the upper part of the figure are benzene rings but in the lower part, the quinol molecules are denoted by a single long sloping line joining the centres of the two hydroxyl groups. [Cf. *J. Chem. Soc.*, 220 (1947)].

cages of roughly spherical shape in the centres of which sulphur dioxide molecules are trapped. The cages are 7.5 \AA in diameter and within this space the sulphur dioxide molecule appears, from the electron density pattern, to be rotating about a line joining the two oxygen atoms. This type of molecular compound (if such it can be called, since there are no chemical forces between the quinol and the trapped molecule) is new to chemistry. It may well occur in a more irregular form in many biochemical systems.

The accurate x-ray analysis of dibenzyl by Jeffrey, mentioned above, was undertaken in the first instance to compare with the crystal structure of geranylamine hydrochloride (17). This is the first compound analysed in detail among the polymerisation prod-

ucts of isoprene, and proves to have a number of interesting characteristics. The two isoprene units are identical within the limits of experimental error, the arrangement of the atoms about the double bond is planar, and most of the bond lengths are normal, as would be expected (Fig. 6). The exception is the carbon to carbon

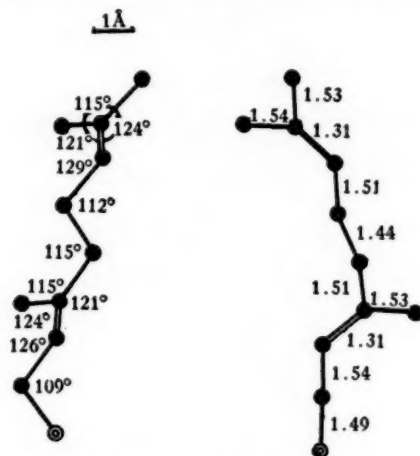


FIG. 6.—Dimensions of the geranylamine ion. Two views are shown.

bond between the two isoprene units where, as in dibenzyl, shortening occurs. This shortening can be explained by the concept of hyperconjugation and may be important in determining the reactivity of isoprene polymers. In the crystal, the carbon chains are packed very roughly parallel to one another and tethered end to end by the ionic interaction between the amino nitrogen atom and the chlorine ion. The electron density contours are close together over the atoms adjacent to the chlorine, but they spread noticeably as the atoms lie further away from this ion, suggesting that here the chain atoms have greater freedom of movement in the crystal. As a whole, the crystal structure is a characteristic layer lattice, so constructed that the coordination sphere of the chlorine ions should be filled by the amino groups and neighbouring carbon atoms, while each amino group is surrounded by four chlorine ions.

One naturally thinks, in connection with layer lattices, of long chain compounds, particularly fatty acids and their salts. The

x-ray analysis of several dibasic acids has been carried as far as electron density projections (18), but still there is no accurate analysis of a typical long chain acid. It is known that a variety of crystal structures are possible and probably the general picture one has of the structures as built up of parallel stacked chains is correct but over simplified. The first soap crystal studied in detail, potassium caprate, shows according to the preliminary account that has appeared (19) at least one interesting deviation from this picture. The zigzag chains lie parallel to one another in single layers, the plane of the zigzag being that of the layer, but succeeding layers are so placed that the chain axes are crossed, making angles of nearly 60° with one another.

So far the analyses of the crystal structures described have depended essentially on the succession of two processes: first, a process of trial to find approximately the atomic positions, followed by electron density calculations, usually repeated several times. The first process is sometimes assisted by the calculation of Patterson or F^2 Fourier series which have the general form:

$$P_{xyz} = \sum_{-\infty}^{\infty} \sum_{-\infty}^{\infty} |F^2 hkl| \cos 2\pi (hx + ky + lz)$$

where the function P_{xyz} has maxima corresponding to prominent interatomic vectors in the structure (20). These series have the advantage that they can be calculated directly from the experimental data and usefully limit the number of possible atomic arrangements to be tried out. But as the crystal structures become more complex, the discovery of the actual atomic positions, even roughly, by any form of trial process, whether assisted by Patterson series or not, becomes more and more difficult. It is for this reason that the crystal structures of the more complex compounds analysed have been mostly of types which make possible, at least a partial, direct calculation of the electron density series without any preliminary trial process. These types are of two kinds. The structure investigated may be analysed in the form of either a derivative containing a very heavy atom, or as a series of isomeric derivatives in which a substituent atom in one of the series is replaced by a heavier atom in another. Provided the heavy atoms make a sufficient relative contribution to the intensities of the x-ray reflections, their positions can be found unambiguously. They can then be used to give evidence about the phase angles,

which varies in completeness according to the symmetry of the crystals.

Perhaps the simplest examples of these processes are to be found in the analyses of nickel glycine dihydrate and copper *dl*- α -aminobutyrate [Stosick (21, 22)]. Both crystal structures are centrosymmetrical and in both the metal atoms are actually at the centres of symmetry. As a consequence, even in Patterson projections, the essential crystal structure is clear—the principal vectors being those between the heavy atoms and the other atoms. The electron density series which could immediately be derived have not been fully refined, but the molecular structures revealed in both cases are of some interest. In both compounds, the metal atoms appear to form coplanar, nearly square bonds to the amino group and to one carboxyl oxygen from each of two amino acid residues. In the nickel complex two water molecules also are attached to the nickel atom so that the metal coordination here is roughly octahedral. The interatomic distances within the amino acid residues are, within the limits of accuracy (which are wide), as would be expected from other amino acid structures (23, 24) (Fig. 7).

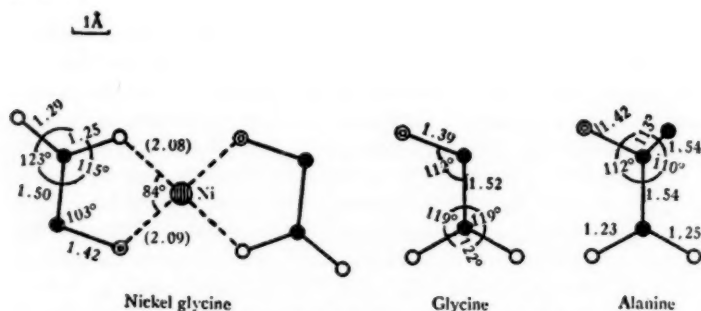


FIG. 7.—Dimensions of nickel glycine, glycine, and alanine.

Another structure analysis, somewhat similar, except that the heavy chlorine atoms are no longer at centres of symmetry, is that reported for 2-amino-4-methyl-6-chloropyrimidine and 2-amino-4, 6-dichloropyrimidine [Clews & Cochran (25)]. Only one electron density projection has yet been obtained for each of these crystals and these show the planar six-membered rings with substituent atoms clearly separated. For the methyl chloro compound, the

electron density peaks representing methyl groups and chlorine atoms are indistinguishable in height, and there is evidently a random distribution of the two about these positions in the crystal structures. This is interesting in view of the well known similarity in the physical properties of many methyl- and chlorine- substituted organic compounds.

Most natural products are however asymmetric. The crystal structures here can never have centres of symmetry and the simple

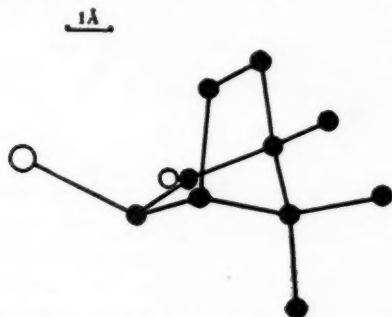


FIG. 8.—Stereochemical form of *d*- α -bromocamphor.

conditions described above do not exist. But certain simplifications are possible in the x-ray analysis if the crystals examined have certain symmetry elements, particularly twofold or twofold screw axes of symmetry. These operate so that projections along the symmetry axes are centrosymmetrical. In the crystal structures of the three derivatives of camphor, *d*- α -bromo, *d*- α -chloro- and *d*- α -cyano-camphor examined by Wiebenga & Krom (26) the crystal unit cells each contain only two molecules related by a twofold axis of symmetry. The positions of the heavy atoms can easily be found and used for the calculation of the centrosymmetrical electron density projections. The projections derived for the three different structures all show clearly an arrangement of peaks in which some, but not all, of the atoms of the camphor skeleton are separately resolved. This peak pattern can only be reconciled with one particular position of the molecule in the crystal and then only if the molecule has a particular stereochemical form, that in which the substituent chlorine or bromine or cyano group is *trans* to the bridge carbon atoms in the camphor formula (Fig. 8). This struc-

ture is confirmed by calculations on x-ray intensities which depend on the parameters of the atom normal to the plane of the centrosymmetrical projection. While these establish the correctness of the structure, they do not permit the accurate measurement of interatomic distances; there is however nothing to suggest that the latter are other than normal.

Very similar crystallographic conditions obtain in the crystal structure of cholesteryl iodide (Fig. 9) (27). Here again one pro-

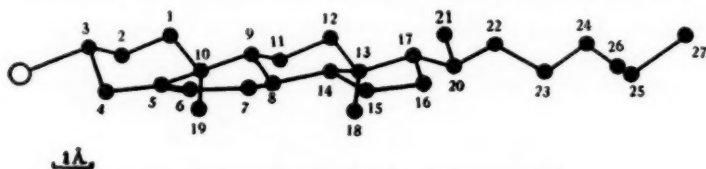


FIG. 9.—Stereochemical form of cholesteryl iodide.

jection can be obtained which is centrosymmetrical and the heavy iodine atom determines the phases required for calculating the projected electron density. Again also, the projection viewed is such that it fixes a large part of the chemical and stereochemical form of the sterol skeleton, which has been checked in three dimensions. The molecule is essentially lath shaped, with the hydrocarbon side chain continuing the general line of the ring system and C₁₇ *cis* to the methyl group at C₁₃. The rings are puckered but there is a general distortion in the region of the double bond at C_{5:6}: all the atoms in its neighbourhood lie in one plane. Ring C is the only one which has a roughly normal Sachse *trans* configuration. Ring D is not planar—and here it may be compared with other five-membered rings studied in camphor, sucrose and penicillin.

So far, in all the structures described it has been possible to recognise the form of the molecule in electron density projections. This was not the case with sucrose, although all three projections were centrosymmetrical and could be derived from the relation between sucrose sodium chloride 2H₂O and sucrose sodium bromide 2H₂O (28). Only a considerable amount of trial comparisons and calculations with different model structures showed the curious way in which the glucose and fructose rings do fit together in the crystal structure. This is shown in Fig. 10, which illustrates the stereochemical relations of all the atoms in the molecule, accurately described as α -D-glucopyranoside- β -D-fructofuranoside. As would

be expected, the glucose ring is puckered and has the Sachse *trans* configuration. The fructose ring also is not planar, one of the carbon atoms, C₄, being out of the plane of the other four atoms. It is not surprising that the packing of molecules of such a shape in the crystal is a somewhat complicated one, particularly since not only the internal bond system of the molecules has to be preserved but also their external relations. Each hydroxyl group in the sugar

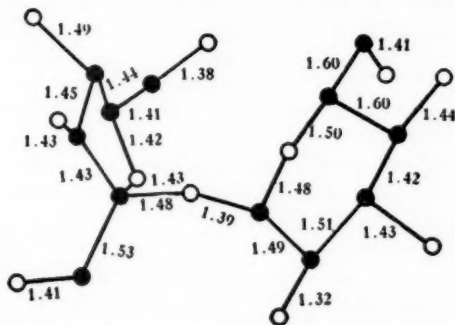


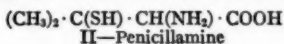
FIG. 10.—Stereochemical form of sucrose in sucrose-sodium bromide-dihydrate. The interatomic distances so far recorded are not accurately determined. They are probably correct to 0.15 Å.

molecule makes in the crystal two external hydrogen bonds, one incoming, one outgoing, and the sodium ions are surrounded by distorted octahedra of bromine ions, water molecules, and hydroxyl groups.

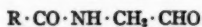
In all discussions on the use of direct x-ray methods to work out chemical structures, one had the fear that something of the conditions seen in the sucrose complexes would obtain and hinder the x-ray analysis. And indeed these fears were quite justified by the appearance of the first direct electron density projections obtained from the isomorphous crystals of potassium and rubidium benzylpenicillin. Here the direct phase determination was far from complete, the first projections therefore very imperfect and all further confused owing to the overlapping of different atoms. How the details of the structure of the molecule were nevertheless derived from these patterns is a story of which only the broad outlines can be given here. The following account is based on the work of Crowfoot, Bunn, Rogers-Low, and Turner-Jones (29) and illus-

trates something of the strategy of the x-ray analysis of a chemically unknown structure.

X-ray measurements were first used in penicillin chemistry as soon as crystalline degradation products of the different penicillins were isolated. Here they were employed chiefly as a means of identification and molecular weight measurement and to follow chemical reactions when these were carried out on too small a scale for the isolation of pure products. But these first degradation products, particularly penicillamine (II) and the penilloaldehydes



II—Penicillamine



III—Penilloaldehyde

(III), were comparatively simple compounds and their formulae were rapidly established by the usual synthetic methods without recourse to detailed x-ray analysis.

With the penicillins, the course taken proved very different, largely owing to the instability of the molecule and the difficulty of its synthesis. Here a knowledge of the structure of the degradation products proved of essential assistance in the actual x-ray determination of the molecular structure. This analysis depended both on the use of an isomorphous series of penicillin salts and partial direct phase determination and also of new rapid methods of testing trial structures, based on the use of optical diffraction. Three salts were used, the isomorphous potassium and rubidium benzylpenicillin, and sodium benzylpenicillin, which had a different but related crystal structure. In the latter case, no atoms were heavy enough to use for direct x-ray analysis and the solution was therefore approached by trying to find an arrangement of one particular type of suggested molecule structure, the oxazolone type, which would give the observed x-ray intensities. Although this form of molecule was far from correct, an arrangement was found which gave quite a considerable degree of agreement with the observations; at this stage, phases were calculated for the trial structure and a trial electron density projection derived.

It was the comparison of this trial electron density projection with the rough electron density projections obtained by direct phase determination from the potassium and rubidium salts that led most clearly to the solution of the structures. For in all these projections, the same molecule was known to appear in roughly the

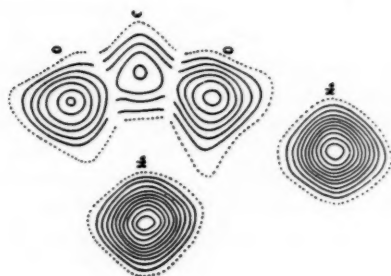
Once the form of the molecule was clear it became easy to recognise in its outlines, seen in the electron density patterns, the positions of certain atoms of the degradation products, particularly the benzyl group and the atoms of the penicillamine skeleton. The positions of these, in turn, determined a number more phase angles and permitted the calculation of new electron density series. The improvement and refinement of these series rapidly became automatic, first in projection, and finally in three dimensions (Fig. 11). And the refinement process itself carried the atoms of the penicillin skeleton into positions in all three crystal structures which could only correspond with one stereochemical form of the β -lactam structure for the molecule (IV).

$$\begin{array}{c} \text{S} \\ | \quad | \\ \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH} - \text{CH} - \text{C}(\text{CH}_3)_2 \\ | \qquad \qquad \qquad | \qquad \qquad \qquad | \\ \text{O}=\text{C} - \text{N} - \text{CH} \cdot \text{COOH} \end{array}$$

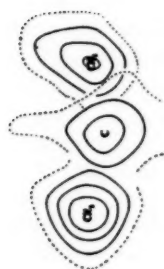
IV—Benzylpenicillin

Beyond the β -lactam ring, there is probably free rotation which will permit the phenylacetyl group to take up various positions in space about the bonds in the molecule. The exact arrangement taken up by this group in the crystal is clearly largely determined

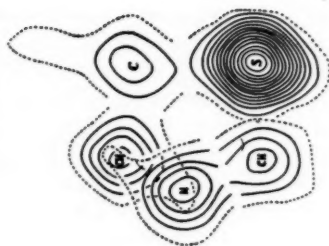
CROWFOOT



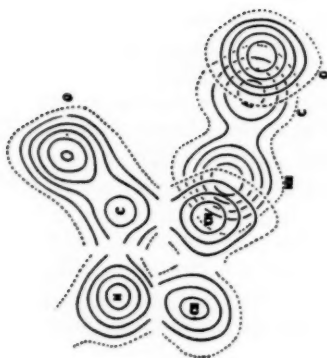
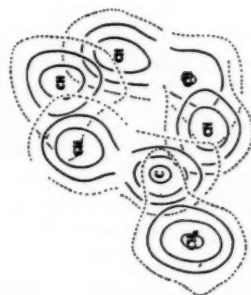
Sodium Ions and Carboxyl Group



Carbon Dimethyl Group



Thiazolidine Ring

 β Lactam Ring

Benzene Ring

X-RAY STUDIES

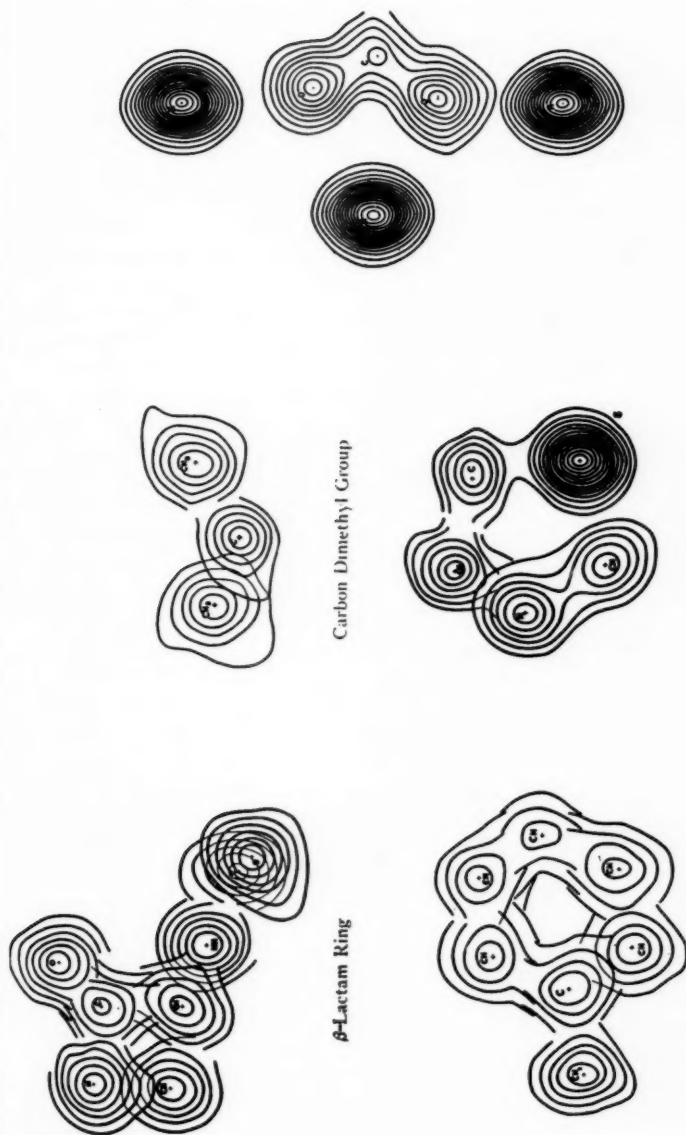


FIG. 11.—The electron density in crystals of sodium and potassium benzylpenicillin. The electron density contours are shown at sections in the crystal passing through the atoms in different parts of the molecule. They are drawn at intervals of 1 electron per \AA^3 , the 1 electron contours being dotted in the case of the sodium salt.

1Å

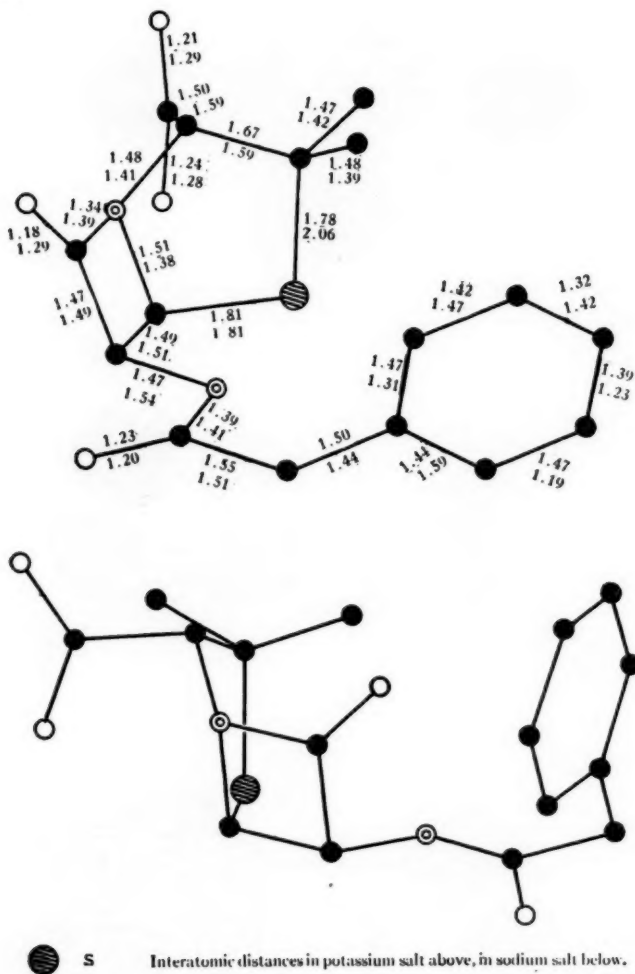


FIG. 12.—Stereochemical form of the benzylpenicillin ion. Again the interatomic distances are not very accurately measured, probably only to within 0.15 Å in the potassium salt, 0.25 Å in the sodium salt.

by packing considerations. It is an arrangement which permits all the oxygen atoms of the penicillin ion, both those belonging to the carboxyl group, the β -lactam oxygen atom, and the amide oxygen atom, to be grouped around the metal ions. All are placed at distances from the metal ions characteristic of the sum of ionic radii and form together a polar layer in the crystal structure. Further, as in inorganic structures, the arrangement changes slightly in the two series, which permits six oxygen atoms only to approach the sodium ion where seven co-operate around the larger potassium and rubidium ions. At the other end of the molecule, all the hydrocarbon parts of the structure pack together to form a nonpolar layer. Again there are alternative methods of packing adopted for the benzene rings, both of them similar to those found in structures such as dibenzyl, for example.

Within the penicillin molecule the bond distances have not so far been measured as accurately as in the simpler structures mentioned earlier, dibenzyl or geranylamine hydrochloride. They are however probably better established than those in sucrose or cholesteryl iodide, where full three dimensional electron density calculations have not been carried out. It seems likely that in these complex structures high accuracy can never be expected: the position of the molecule as a whole in the crystal may vary within the accuracy required for determination of bond character. This is indicated, indeed, by the number of x-ray reflections that can be observed and used in the analysis. For geranylamine hydrochloride, 1060 reflections were observed and used to place 11 atoms in three dimensions, in potassium benzylpenicillin the corresponding numbers were 650 for 24 atoms, in cholesteryl iodide, 301 for 28 atoms. Nevertheless, the present accuracy in the last two structures, somewhere of the order of ± 0.1 to 0.2 Å, is quite sufficient to determine the positions of the atoms in the molecules and hence the arrangement of the bonds between them.

THE X-RAY CRYSTALLOGRAPHIC STUDY OF MACROMOLECULES

There is strictly no gap in the series of known organic compounds between the group of substances considered in the last section and those now to be discussed. But there is a pronounced gap in the series of compounds yet studied by x-ray methods. The largest of those mentioned in the last section, cholesteryl iodide, has a molecular weight of nearly 500. The smallest molecular

weight proteins yet examined, such as ribonuclease, have molecular weights of the order, at least, of 12,000. And these are probably smaller than most of the macromolecules to be discussed in this section.

From a crystallographer's point of view it is convenient to distinguish the class of fiber structures from those in which the crystal unit cell itself contains macromolecules. In fiber structures, the unit most easily identified is a part only of the molecule. Also, at right angles to the fiber axis, the number of these units is usually small or their alignment imperfect, so that the x-ray reflections they give tend to be few and diffuse, very unlike the sharp diffraction effects from perfect crystals. But while it is easy to preserve the distinction between typical fiber structures such as cellulose, and typical large molecule crystal structures, such as haemoglobin, the two classes become confused to some extent, as we approach more complex biological products such as muscle.

The most detailed recent analysis of any of the simple fiber structures is probably that carried out on two nylon structures, polyhexamethylene adipamide and sebacamide, 6.6 and 6.10 polyamides [Bunn & Garner (30)]. In both fibers two crystalline forms are found, with the α -variety predominating, sometimes exclusively. In this form only one chain molecule passes through the crystal unit, and the fiber axis repeat corresponds to a fully extended zigzag chain. The atomic positions can be found by trial and error methods with reasonable certainty though not accurately enough to warrant the recording of interatomic distances. From the analysis the nylon chains are found to be planar or very nearly so, with the oxygen atoms, only, removed a little from the plane of the chain. The molecules are linked together by hydrogen bonds between the CO and NH groups of adjacent chains to form sheets (Fig. 13). This necessitates a rather open packing of the methylene groups in the chain, which appears to be partly compensated for by a slight turn of the chain carbon atoms from the plane of the hydrogen bonds and partly by the relative packing of the chains in two succeeding sheets. The second type of crystal structure differs from the first chiefly in the packing of the sheets; sometimes very disordered structures appear in which the alternative arrangements are adopted, more or less at random, throughout the fiber. The whole arrangement is of particular interest in relation to the structure proposed for the β -keratin group of fibrous proteins. The

x-ray reflections given by the latter are far too limited to permit the placing of atoms within the unit. But the strong reflection at about 4.5 Å, observed in these structures, and considered by Astbury to correspond to the backbone spacing of the peptide chains, is extremely similar to that of 4.4 Å in the nylon structures, which is here established as having exactly the postulated relation to the

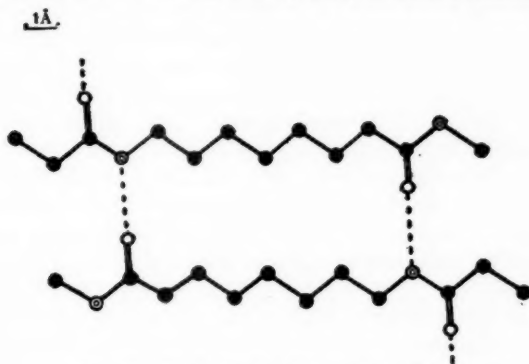


FIG. 13.—Hydrogen bond system found in nylon.

chain system. And further the relative orientation of the planes having 4.4 to 4.5 Å spacing in pressed samples, both of nylon and of β -keratin (in the form of horn), is the same. In both, these planes are normal to the plane of the pressed strips, i.e., the hydrogen bonds in nylon, and all the more probably also in β -keratin, lie in the main planes of the strips.

The principal difficulty in confirming the extended chain configuration for β -keratin from x-ray data in the past has certainly been due to the relatively very small number of x-ray reflections available, entirely insufficient to establish the structure. This in turn has been partly due to the variable character of the amino acid groups in the chain. As such, it should be possible to obtain some direct proof of the structure by the examination of synthetic peptides containing only one type of amino acid residue. Several of these are now available and more detailed x-ray study of one or more of them is certainly desirable.

Both the limitations and possibilities of x-ray data on fiber structures are well illustrated by recent work on polysaccharides. Of the main group of those investigated, all, from chemical evi-

dence, have pyranose rings joined by glycosidic oxygen atoms. If these ring systems are to form an extended chain, then models show there are at least two alternative stereochemical ways of achieving this end, while preserving, as unit, a Sachse *trans* form of the pyranose rings. In one of them, Type I, the bonds joining C₁ and C₄ to the glycosidic oxygen atoms make an angle of about 20°, and in the other, Type II, about 90°, to the 'plane' of the ring system as a whole. The fiber periods calculated, are, in the one case, 5.15 Å, per ring system, in the other, 4.37 Å [Astbury (31), Palmer & Hartzog (32), Palmer & Lotzkar (33)]. Comparison with the actual fiber periods found in a series of polysaccharides suggests that both forms occur in nature, and moreover, that they are interchangeable. Thus cellulose has a fiber period of 2×5.15 Å and clearly has configuration I. But various cellulose derivatives have fiber periods that may well be multiples of 4.37 Å; the period of 25.6 Å for trinitro-cellulose, for example, is more likely to be 4.37×6 than 5.15×5 Å. Alginic acid, with a fiber period of 8.7 Å, belongs, on the other hand, to type II, while sodium alginate (fiber period, 15 Å) appears to belong to type I. Both pectin and sodium pectate, which are based on α-galacturonic acid residues, belong to type II and associate the fiber period of 13.1 Å with a threefold screw repeating unit.

Relatively more complex types of chain configuration and fiber period are found among the starches. And here an interesting attempt at a direct x-ray analysis of V-amylose¹ has been made by Rundle (34), using essentially the methods already described, the study of a heavy atom derivative. V-amylose, precipitated from the amylose fraction by alcohols, absorbs iodine vapor rapidly up to 26 per cent of its own weight. The product gives a good x-ray powder pattern which can be indexed on a hexagonal lattice, isomorphous with that shown by V-amylose itself. The character of the lattice suggests that the glucose residues are arranged in a sixfold helix, of external diameter roughly 17 Å, and height 7.9 Å. In the column within the helix, the iodine atoms or molecules may be placed. This proposed structure agrees with the very rough electron density patterns obtained, first from V-amylose-iodine complex and then from V-amylose itself. In the first, the phase angles used are based on the iodine atom positions alone. The pro-

¹ Starch and amylose precipitated by alcohol have a characteristic x-ray pattern known as the V type.

jection shows the expected very heavy peak corresponding to the column of iodine atoms in projection, surrounded by a ring of six diffuse maxima, in roughly the expected positions for the glucose residues. For V-amylose itself, the pattern derived shows, rather more clearly defined, the six maxima in the helix. But in neither case is there anything approaching the resolution of the electron density patterns necessary to show individual atoms: only sixteen x-ray reflections were available even in the iodine complex, far too few to use to determine atomic positions. And this is likely to be the case with many polysaccharide polymers. Only very well oriented fibers can give the requisite number of reflections to resolve atoms. But it still seems interesting to determine, even roughly, the position of the glucose residues in these polymers, and there are other groups in which similar x-ray analytical methods can be applied, for example the alkali amyloses which contain lithium, sodium, potassium and caesium hydroxide [Senti & Witnauer (35)].

The conditions for the x-ray analyst dealing with crystalline proteins are very different from those found in fiber structures and yet there are certain similarities. These are very well illustrated by the study of horse methaemoglobin carried out by Boyes-Watson, Davidson & Perutz, of which a full account has just appeared (36). In the crystal unit cell of horse methaemoglobin, there are two protein molecules whose weight, accurately measured from the x-ray data, is 66,700 (± 3500). There is also liquid of crystallisation, the quantity of which varies according to external conditions from 52.4 per cent by volume in the normal wet crystals to approximately 10 per cent in air dried crystals. The number of atoms in the unit cell is consequently variable; but in each protein molecule alone there are roughly 5000 atoms (not counting hydrogen). These molecules are not, however, the crystallographic asymmetric unit; since they are situated on a twofold symmetry axis, the crystal unit is a half molecule, and the number of atoms which have to be placed to solve the structure is 2500. Large numbers of x-ray reflections are needed for this solution, and in fact, some 4500 have been recorded (compare the sixteen mentioned above for V-amylose). With so many reflections, calculations of the usual type become exceedingly tedious, and yet there are not actually enough reflections observed to determine the individual atomic positions, when we recollect the relative numbers of reflections to atoms used in, say, the structure analysis of penicillin. Further, the

relative scattering contribution of so many atoms is so great, that it is clearly impossible to determine the phases of the reflections from haemoglobin by introducing one or even a few heavy atoms into the crystal.

The attack on the solution of the crystal structure has been made therefore in rather different ways, principally through a study of the variation in amount and composition of the liquid in the crystals. In the first place, the composition of the liquid may be changed, keeping the cell dimensions constant, by growing the crystals in various concentrations of neutralized ammonium sulphate and potassium phosphate solution and also water itself. In this way, crystals were grown in which the salt concentration of the liquid present varied widely. Determination of the crystal density and of that of the solution in which the crystals had been grown showed that the density of the liquid of crystallization is always less than that of the suspension liquid. This suggested to Perutz the presence of liquid in the crystals not diffusible to ions—'bound' water—and it is interesting that the weight of such water, calculated from the experimental results, corresponds roughly to the weight of a layer one molecule thick over the surface of the protein molecule (37).

If the hydrogen ion content is changed, or the crystals gradually dried, the crystals change in other ways. For example, in ammonium sulphate and ammonium phosphate solutions they expand suddenly to a new form at pH 5.4; on drying they shrink, stepwise, to an air dried state, which again varies a little in cell dimensions according to the exact experimental conditions. The changes are reversible and discontinuous, and all involve changes principally in one crystallographic direction. They indicate a relatively rigid structure of layers of haemoglobin molecules which can move by shearing over one another. Between them lies the liquid of crystallization in a single layer and the thickness of this layer varies in four of the shrinkage stages studied by the intervals, 4.2, 4.6, 3.8 and 6.3 Å, the total difference between the air dried state and the most expanded state examined, being 18.9 Å, and between the former and the normal state, 15 Å. The sizes of these intervals suggest that the thickness of the water layer changes by one layer of water molecules at a time, possibly by two layers in the final shrinking to the air dried state. The actual thickness of the layer differences is rather greater than the thickness of 3.7 Å for a

puckered hexagonal layer of water molecules in the crystal structure of ice, but probably the presence of ions in the liquid accounts for this difference.

In the above deductions various arguments, based on the detailed characteristics of the x-ray data, played a part, and particularly the changes in the intensities of the x-ray reflections. These could be used in several ways, both to throw more light on the shape of the molecules and on their internal structure. Methods of calculation had first to be devised for dealing with relatively large volumes of scattering matter, considered as roughly uniform in density. Having these, both trial and error calculations were made and also direct electron density calculations based on phases deduced through the changes in the density of the liquid of crystallization. These were further cross-checked by an attempt to trace the actual scattering effect of the individual molecules in the different crystals prepared. These calculations all led to a somewhat modified picture of the structure of haemoglobin from that earlier suggested. They fit most closely with the view that the molecule is cylindrical in form, of height 36 Å and radius 28.5 Å. And within this cylinder, parallel to the base, the electron density patterns indicate the concentration of the scattering matter inside the molecule in four layers, 9 Å apart, which was described earlier.

The variations in the intensities of the x-ray reflections and the peak structure of the derived Patterson series indicate that there is still far more to be learned from the x-ray data than this. But clearly also the x-ray data are insufficient to give resolution of the electron density patterns, even if these could be fully calculated, into actual atomic positions. Such resolution does not occur experimentally unless the spacing limit of reflections observed is of the order of 1.2 Å and so far, in haemoglobin, the spacing limit is of the order of 2.5 Å. Considerations of this kind might make us pessimistic about the possibilities of ever testing the reliability of x-ray evidence on such complex structures as proteins, were it not that direct confirmation of certain of our conclusions has already been reached through direct electron microscope observations of crystalline viruses.

The direct vision of the elementary particles of a number of viruses, and of these particles as they are arranged in crystals, has been one of the most remarkable developments of the last three years (38). So far, two fully crystalline viruses, tomato bushy stunt

and tobacco necrosis virus (Rothamsted strain), have been examined both by x-rays and by the electron microscope, and certain others, the southern bean mosaic virus and other strains of tobacco necrosis virus, only, as crystals, by the electron microscope (38). In the case of tomato bushy stunt virus, the diameter of 276 Å calculated from x-ray photographs for a spherical molecule (39) agrees reasonably well with the diameter 255 to 270 Å of the spherical particles actually seen in the electron microscope pictures (40). In the pictures so far taken, these particles are usually arranged in hexagonal close packed layers, but in small regions the beginnings of crystals can be traced, and here it does appear that the particle arrangement changes as indeed is required by the body centered packing found in the crystal. These changes suggest some quite specific orienting forces operating in the formation of the crystals, and evidence of a similar kind can be deduced from the crystals of the tobacco necrosis viruses. There are several strains of these viruses which contain particles differing in molecular weight. The crystals derived from the Rothamsted strain are, remarkably enough, triclinic, and the x-ray photographs show that order is maintained, at least to a similar degree to that found in crystals of smaller protein molecules (41). Evidence of sharp x-ray reflections with spacings as small as 2.8 Å was obtained although the estimated diameter of the particle is 160 to 200 Å. One at least of the larger molecular weight tobacco necrosis viruses also appears to have a triclinic structure from the electron microscope pictures. Here the particle diameter is about 275 Å (42).

That there were large particles, even approximately spherical particles, in these virus crystals was fairly clear from x-ray data, even without the direct confirmation from the electron microscope. But the interpretation of the x-ray reflections of long spacing observed with many natural protein fibers has long been a matter for controversy. Here the x-ray data are very much less extensive; a lower order of regularity of molecular arrangement all together is involved. But the very magnitude of the x-ray spacings observed in structures such as feather keratin and porcupine quill tip suggests that the units involved, corresponding to the pyranose groups of a polysaccharide chain, may be molecules or corpuscles as large as proteins or viruses. Some confirmation has now been obtained for this view from electron microscope pictures but the situation is still very complex (43).

Some of the best experimental evidence from the x-ray side has been described by Bear (44). Bear re-examined both wide and small angle x-ray patterns from feather keratin, porcupine quill and paramyosin fibers from clam muscle, all of which belong to the keratin-myosin group of Astbury. The patterns from these structures form a series of increasing complexity. The fiber periods of the three are 95, 198, and 725 Å, the strongest meridian spacings, 24, 66, and 145 Å, and the largest lateral spacing, 34, 83, and 325 Å respectively. The last two, in addition, give α -keratin wide-angle patterns, the first, β -keratin like. These can be fitted into crystallographic relation with the small-angle series, but naturally the relations vary widely from structure to structure. The small-angle pattern from paramyosin is particularly interesting and complex and this has received direct confirmation from electron microscope studies. Hall, Jakus & Schmitt (45) found that phosphotungstic acid combined with specific regions in the fibers, forming a regular geometrical pattern which was visible in the electron microscope. The meridian spacings in this pattern agreed excellently with those observed by x-rays; the lateral spacing, however, was a good deal smaller, 193 instead of 325 Å, possibly owing to the somewhat drastic conditions of observation. The whole appearance of the pattern is strongly suggestive of regular packing of large particles, but there is no evidence here of three dimensional regularity. The fibers themselves are ribbon-like in shape, and at right angles to the plane of the ribbon the "particles" may either be too small or too large for observation.

The relation that the paramyosin structure has to the properties and function of muscle as a whole is quite obscure. The electron microscope pattern is observed whether the fibers are prepared from extended or contracted muscles, and further not all muscles contain paramyosin fibers. The most interesting feature of their structure is the evidence it provides of quite complex organization of large units into fibers. This organization is perhaps even better illustrated in a dynamic way by the properties of tropomyosin and actin.

Tropomyosin is a remarkable protein which can be isolated from the fibrillar fraction of skeletal and cardiac muscle [Bailey (46)]. It crystallizes in large, birefringent crystals, which contain 90 per cent of water of crystallization. Ultracentrifuge and osmotic pressure measurements indicate a molecular weight of 90,000.

When the material is dissolved in water, however, the solutions are extremely viscous and electron microscope observation shows a mesh of thin fibrils. Thin films of the protein are clearly also fibrous in character; they give the α -keratin diffraction pattern which on heating or pressing transforms into the β pattern. Yet if the protein is transferred to 0.1 M KCL, the viscosity of the solution falls, flow birefringence disappears, and no trace of fibers can be observed in the electron microscope.

Very similar properties are shown by the protein actin, discovered as an essential muscle constituent by Straub (47). This exists as globular G-actin which is converted by reversible linear aggregation to the fibrous form, F-actin, by lowering the pH with acetic acid or adding potassium chloride at pH 7. The transformation has been studied in the electron microscope by Jakus & Hall (48) and by Astbury *et al.* (49). Further the x-ray patterns obtained by Astbury from F-actin (50) show long spacings, nearly identical with patterns obtained both by Bear from his so-called type II muscle fibrils (51) and by Astbury from frog sartorius muscle, but with one important difference. F-actin does not show the α -keratin wide angle diffraction pattern found in the muscle patterns. This suggests to Astbury that the characteristic feature of the actin-myosin combination is the combination of a corpuscular with an elastic fibrous protein, the state of folding of the myosin partner being stabilized by the parallel actin system. The loosening of the combination between actin and myosin by ATP certainly plays an important part in muscle contraction, though the mechanism involved is still a subject for controversy (52, 53) and for further experiment.

The picture we have here is of fibrous structures of various orders of complexity. Within the corpuscular units of actin and tropomyosin, and also within the large scale structures of keratin and collagen, there is an arrangement of amino acids which appears in many ways to be fibrous in character and which is mainly responsible for the wide angle scattering. The corpuscular units may then themselves either pack together or actually be fused together by chemical polymerisation. The latter may be the state in structures such as feather keratin or porcupine quill. And in addition, in biological systems, a cement of the elementary peptide fiber structure may also be present. So we have systems of fibers within corpuscles and corpuscles within fibers, as Bernal has said,

and both combined in more ways than one to make structures to the complexity of which x-ray studies can do little more than act as a pointer.

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CHEMISTRY OF THE LIPIDS¹

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CHEMISTRY OF COMPLEX LIPIDS

Nomenclature of phosphatides.—Progress in the study of phosphatide chemistry in recent years has rendered obsolete the customary classification into monoaminophosphatides (lecithin and cephalin) and diaminophosphatides (sphingomyelin). The terms lecithin, cephalin, and sphingomyelin have been used indiscriminately to refer either to definite chemical compounds, or to phosphatide fractions defined by their solubilities. This custom has been based on the assumption that lecithin, the fraction soluble in ether and alcohol, was diacylglycerophosphoryl choline; that cephalin, the fraction soluble in ether and insoluble in alcohol, was diacylglycerophosphoryl ethanolamine; and that sphingomyelin, the fraction soluble in hot alcohol, was N-monoacylsphingosophosphoryl choline. This practice is the source of endless confusion for the following reasons: (a) Besides the three compounds enumerated above several new phosphatides have been isolated and identified during recent years; (b) Fractions from different sources, defined only by solubility, may have widely differing compositions. For example, brain cephalin is a mixture of three different phosphatides (1), while egg cephalin is fairly pure diacylglycerophosphoryl ethanolamine and soy bean cephalin appears to be a mixture of several uncharacterized phosphatides; (c) Even from one single source fractions defined by the same solubility can exhibit differences in composition, according to the intermediary steps of the procedure of isolation, because the solubility of one pure phosphatide is greatly modified by the presence of others.

For these reasons the reviewers advise against the further use of the terms lecithin, cephalin, and sphingomyelin, except possibly

¹ This review covers the period from December, 1946 to December, 1947.

for crude fractions, and propose the adoption of the names "phosphoglycerides," "phosphoinositides," and "phosphosphingosides" to designate, according to chemical composition, the three groups of phosphatides known at the present time. Under this scheme of nomenclature the "phosphoglycerides" would include the class of compounds having as a constituent the diacylglycerophosphoryl radical, namely phosphatidic acids, phosphatidyl choline (lecithin), phosphatidyl serine, and phosphatidyl ethanolamine. The structure assigned by Pangborn (2) to cardiolipin would place it in this group. The name, "phosphoinositides," would be applied to lipids with both inositol and phosphorus as constituents. At least four, and probably five, such phosphatides are known, namely, diphosphoinositide from brain, lipositol from soybean, an inositol-containing phosphatide recently isolated from soybean, which appears to be different from lipositol, and at least one, and probably two (3) phosphoinositides from tubercle bacilli. The term "phosphosphingosides" would be used to designate phosphatides having sphingosine as a constituent (sphingomyelin), and would be a subgroup of the "sphingolipids" (4). As the structure of the phosphatides containing higher fatty aldehydes (plasmalogen) is not yet known with certainty (5), they can not be fitted into the proposed system of nomenclature at the present time.

Phosphoglycerides.—A major development in this field was the synthesis of dipalmitoglycerophosphoryl ethanolamine (cephalin) by Rose (6). This was accomplished by two different new methods: in one, dipalmitoglycerophosphoryl chloride was treated with carbobenzoxyethanolamine and the "carbobenzoxycephalin" thus obtained was cleaved with phosphonium iodide. In the second, dipalmitoglycerophosphoryl chloride was treated with β -hydroxyethylphthalimide and the "phthalylcephalin" thus obtained was cleaved with hydrazine. The over-all yield was about 25 per cent of the dipalmitin used. The product is microcrystalline and melts at 193°–194° or 195°–198° according to the rate of heating. By the method of Kabashima (7) only a small amount of material, melting at 77°, was obtained. The constitution of the compound obtained by both new methods was confirmed by its quantitative reaction with nitrous acid and by the composition of a 3, 5-dinitrobenzoyl derivative. While this synthetic phosphatidyl ethanolamine is not exactly comparable to the natural products, which are known to have unsaturated acids as constituents, its synthesis represents a

and 3 per cent sulfur, as compared with 6 per cent at pH 9. "Lecithins" reacted to give compounds containing 1.7 to 2.3 per cent sulfur.

Burmester (14) applied his micromethods for the estimation of α - and β -glycerophosphoric acids to the study of a number of phosphatide fractions. He found that both α - and β - "lecithins" and "cephalins," separated by the methods of Suzuki, Nishimoto & Yokoyama (15 to 18), yield the same mixture of α - and β -isomers of glycerophosphoric acid, and thus he showed the method of the Japanese workers to be unreliable.

Phosphoinositides.—The inositol-containing phosphatide, shown to be present in brain "cephalin" (19), was isolated (20), and the name of "diphosphoinositide" was given to it. While its complete structure has not yet been established, it was found that the constituent inositol is present as inositol diphosphate and that the two phosphoryl radicals on the inositol molecule are in the meta-positions. This fact was demonstrated by reaction of periodic acid with the inositol diphosphate isolated from the cleavage products of diphosphoinositide. It was found that two moles of periodic acid are used and one mole of formic acid is produced per mole of inositol diphosphate. One mole of inositol-*p*-diphosphate would have used two moles of periodic acid without any production of formic acid, while one mole of inositol-*o*-diphosphate would have used three moles of periodic acid with production of two moles of formic acid.

It was shown further that diphosphoinositide contains one mole of fatty acid and one of glycerol per mole of inositol diphosphate. It is an acidic phosphatide and was isolated as a magnesium calcium salt. It contains 0.6 per cent nitrogen (all as amino nitrogen), but it is still doubtful whether the amine present is a component or an impurity. The name of diphosphoinositide was chosen to emphasize the difference between this compound and other known phosphoinositides in which inositol and phosphoric acid are present in equimolar amounts.

A second phosphoinositide was isolated from soybean (21) and shown to contain inositol, phosphoric acid, carbohydrate, glycerol, fatty acids, and an unidentified amine. It appears to be different from lipositol (22) which contains tartaric acid and no glycerol. The ratio of inositol to phosphorus is equimolar and therefore this phosphatide belongs to the general group of monophosphoinositides.

The presence of inositol as a constituent of liver phosphatides was reported by Mcpherson & Lucas (23). The concentration of inositol in the preparations so far described was less than that found in phosphoinositides from brain and soybean, and the solubility characteristics also appeared to be different.

De Suto-Nagy & Anderson (3) studied the polysaccharide components of phosphatides liberated by mild alkaline hydrolysis from two lots of tubercle bacilli which had been grown under identical conditions from what was thought to be the same strain. One lot gave inositol monophosphoric acid, glycerophosphoric acid, and a glycoside which contained phosphorus, inositol, and mannose. The other, though worked up by identical procedures, yielded glycerophosphoric acid, inositol glycerol diphosphoric acid, and a glycoside which on hydrolysis gave glycerophosphoric acid, inositol, and mannose. The phosphatides from each lot gave about 70 per cent of fatty acids. These results indicate that different phosphoinositides were present in the two lots of tubercle bacilli. The inositol glycerol diphosphoric acid from the second lot yielded approximately equimolecular quantities of inositol and glycerol on hydrolysis with 10 per cent sulfuric acid in a sealed tube at 160°, and inositol monophosphoric acid and glycerophosphoric acid on hydrolysis by 10 per cent sulfuric acid at 45° (24). These findings were interpreted as showing that the molecules of inositol and glycerol are combined either as esters of phosphoric acid or by an easily hydrolyzable oxygen bridge. Lack of material prevented further investigation of the chemical constitution.

Sphingolipids.—This name was proposed by Carter *et al.* (4) to designate the group of lipids of which sphingosine is a constituent. It includes not only phosphosphingosides (sphingomyelin), but also the following known nonphosphatides: the cerebrosides, for which the general term "glycosphingosides" and the specific terms "galactosphingosides" and "glucosphingosides" are proposed; gangliosides; and possibly cerebron-sulfuric acid (25).

Carter *et al.* (4) prepared sphingolipids from spinal cord and brain by a procedure which gave better yields than have been obtained by other methods. The tissue was dehydrated with acetone, the phosphoglycerides were removed with ether, and finally the sphingolipids were extracted with boiling ethanol and precipitated from the ethanolic extract in the icebox. Dihydrosphingosine was isolated from the cleavage products of these sphingolipids (26). It appeared to be more abundant in spinal cord than in brain. Dihy-

drosphingosine was previously isolated from *Cysticercus fasciolaris* (12), but its presence has not been shown before in the central nervous system. In the course of an exhaustive study of the chemistry of dihydrosphingosine, Carter *et al.* devised methods for the preparation of sphingosine and dihydrosphingosine, and their N-acetyl, N-benzoyl, triacetyl, and tribenzoyl derivatives.

Carter *et al.* (27) confirmed and expanded their previous work on the structure of sphingosine (28). They found that on catalytic reduction triacetylsphingosine undergoes partial hydrogenolysis of an acetoxy group; a finding which strongly indicates that one of the acetoxy groups is adjacent to a double bond. By the lack of reaction between periodic acid and either N-acetyl or N-benzoyl dihydrosphingosine they proved the absence of a 1,2-glycol group. They established the 1,3-glycol structure by preparing the benzylidene derivative of N-benzoyldihydrosphingosine. This reaction is characteristic of 1,2 and 1,3 glycols; the former of these two possibilities was excluded by the lack of reaction with periodic acid. Finally, they studied the products of the reaction of dihydrosphingosine with periodic acid and found them to be palmitaldehyde, formic acid, ammonia, and formaldehyde. These findings establish that the structure of sphingosine is 1,3-dihydroxy-2-amino-octadecene-4.

Carter *et al.* (29) explored the possibility of reducing α -amino- β -hydroxy esters by Raney's nickel catalyst as a step towards the synthesis of compounds of the sphingosine type. They found that the methyl esters of DL-allothreonine and DL-threonine are reduced smoothly to 1,3-dihydroxy-2-amino-butane, the former yielding a single isomer, and the latter giving a mixture of two possible isomers. By the same method, methyl α -aminostearate was reduced to 1-hydroxy-2-amino-octadecane.

Schmidt *et al.* (11) applied their new method (see below) to the determination of phosphosphingosides (sphingomyelin) in several organs of various animal species. There appeared to be a considerable species difference; e.g., the sciatic nerve in cats contained over twice as much as in rats. However, in both species the content of phosphosphingosides was much higher in the sciatic nerve than in the brain. In beef brain the white matter contained much more than the cortex. It is of interest that the concentration of phosphosphingosides in the rat kidney was almost twice that of the brain.

General studies of phosphatides.—In a careful analysis of serum phosphatides, Sinclair (30) obtained in some animal species data

which were close to the theoretical values for phosphatidyl choline. In other species the presence of some "cephalin" was indicated.

Hanahan & Chaikoff (31) studied conditions for complete extraction of phosphatides, both from raw and steam-treated carrots. They found that carrot phosphatides are characterized by low nitrogen content and either absence or low content of choline. Phosphatides isolated from steam-treated carrots were richer in choline than those obtained from raw carrots. The presence in carrots of a phosphatide-splitting enzyme, which appears to be specific for the ester linkage between the nitrogenous base and the phosphoric acid, was demonstrated (32). This enzyme has a maximal activity between pH 5.2 and 5.9 in 0.05 *M* phosphate buffer. It is fairly thermostable, not being completely inactivated when exposed to a temperature of 95° for fifteen minutes. The presence of this enzyme explains the earlier finding (31) of a lower content of choline in phosphatides isolated from raw carrots than in those isolated from steam-treated carrots.

Quantitative methods.—Schmidt *et al.* (11) developed a method for the quantitative estimation of phosphosphingosides (sphingomyelin) in lipid mixtures. The procedure is based on the fact that phosphosphingosides are not affected by treatment with normal sodium hydroxide at 37° while phosphoglycerides release their phosphorus quantitatively as acid-soluble phosphorus under the same conditions. Thannhauser *et al.* (33) applied this principle on a macro scale to the development of two methods for the preparation of pure phosphosphingosides from lung. Palmitic and lignoceric were the only fatty acids whose presence could be detected in such preparations.

Hack (34) studied in considerable detail the specificity of the reineckate procedure for the determination of phosphosphingosides. He isolated phosphosphingosides, glycosphingosides, and "cephalin" fractions from the brains of various animal species, and showed that all of these preparations would form reineckates, of which those prepared from crude extracts contained both glycerol and hexose. Furthermore, quantitative recovery of pure phosphosphingosides as the reineckate could not be achieved. Hack obtained evidence indicating that the ammonium reineckate-phosphosphingoside precipitate is an adsorption complex and not a true chemical compound. It seems evident from this careful investigation that the reineckate method is not suitable for the determination of phosphosphingosides in brain tissue. Hack also reported

that the assay of pure phosphosphingosides for choline gave low values, which he ascribed to the difficulty in hydrolyzing the phosphoryl-choline bond.

Sperry & Brand (35) studied conditions for complete hydrolysis of phosphosphingosides (sphingomyelin) previous to choline determination by their micro-procedure (36). They obtained evidence that hydriodic acid will cleave choline from "sphingomyelin" completely in three hours at 137° in sealed tubes, but it has not been possible to apply this reaction satisfactorily to the estimation of choline in brain lipids.

Burmester (37) described micromethods for the estimation of α - and β -glycerophosphoric acids in hydrolysates of phosphatides. He made use of the well-known reaction of periodic acid with 1, 2-glycols to produce glycollic aldehyde phosphate from α -glycerophosphoric acid (38) without any decomposition of the β -isomer. After reaction with the hydrolysate, as carried out by earlier workers, the unused periodic acid and the iodic acid produced are eliminated with sulfite, the glycollic aldehyde phosphate is hydrolyzed with hot acid, and the resulting orthophosphate is estimated colorimetrically. By this ingenious modification Burmaster rendered the method specific for α -glycerophosphates in the presence of serine, ethanolamine, and inositol, which also react with periodic acid. For the determination of total glycerophosphoric acid phosphorus the above procedure is applied after treatment of another portion of the sample with hot acid, which was shown by Bailly (39) to transform all glycerophosphoric acid to the α -isomer. Findings on the α - and β -isomers of glycerophosphoric in chemical hydrolysates can be translated to the original phosphatides only with considerable reservation because of the possibility of migration of the phosphoryl radical (40) during the hydrolysis. Although glycerophosphoric acid isomers have been proved to be stable on alkaline treatment (41), Bailly & Gaumé (42) showed that on alkaline hydrolysis of the methyl ester of either α - or β -glycerophosphate such migration occurs, so that a mixture of two-thirds β - and one-third α -glycerophosphate is always obtained. Although Burmaster (14) presented some indirect evidence that migration of the phosphoryl group did not occur under the conditions of hydrolysis he employed, he was unable to exclude the possibility.

Periodic acid has been known for some time to liberate ammonia from serine (43), ethanolamine (44), and specifically from hydrolyzates of "cephalin" (44). Burmaster (45) applied this reaction

to the determination of serine and ethanolamine in phosphatides.

Hack (46) developed methods for the analysis of phosphatides in blood. From the total lipid phosphorus, the phosphorus liberated in acid-soluble form by hydrolysis with *N* KOH at 37° for sixteen hours, and the choline released under the same conditions, the concentrations of "lecithin," "cephalin," and "sphingomyelin" are calculated. With this procedure Hack confirmed the low content of "cephalin" in plasma lipids, first shown by Folch, Schneider & Van Slyke (47) and more recently by Sinclair (30).

FATTY ACIDS AND TRIGLYCERIDES

Reviews.—A second edition of Hilditch's valuable book on the constitution of fats was published (48). Hilditch (49) also reviewed the historical development of fat technology. In critical reviews (50, 51) of the commercial production of edible fat from hydrocarbons in Germany it was pointed out that these synthetic fats contain C_{10} to C_{20} fatty acids with odd and even carbon chains in about the same proportion, and that branched chain, hydroxy, keto, and dicarboxylic acids of questionable nutritional value are present. The usual complete annual review of the literature on fats and oils was compiled by Piskur (52). Reviews on fat spoilage (53), amines of higher fats (54), fat splitting (55), and the structure of glycerides in fats (56) were not available to us, but are listed for their possible value to others.

Composition of natural fats.—Hilditch and his collaborators continued their comprehensive analyses of natural fats with the practical objective of finding new sources of commercially useful oils. The seed oil of *Tetradlea carinata* contains more linolenic acid (64 to 68 per cent) than linseed oil and 75 per cent of the glycerides contain two or three linolenic acid groups (57). In Australian lumbang (candlenut) oil 32 per cent of the glycerides contain only linoleic and linolenic radicals (58). West Indian ben oil is similar to olive oil in having a very high content (about 75 per cent) of oleic acid, while mango oil has a high content (43 per cent) of stearic acid (59). The component fatty acids of "pique-a" fruit coat fat are almost entirely palmitic and oleic in about equal proportions, and the component glycerides are mainly oleodipalmitin and palmitodiolein. No triolein is present and the mono-oleo glycerides consist entirely of β -oleodipalmitin. Both symmetrical and unsymmetrical palmitodiolein are present (60). The procedure of fractional crystallization at low temperatures (61) was ap-

plied to reinvestigations of the composition of palm (62), soybean (63), and rape oils (64). The presence of 2.4 per cent of linolenic acid, not reported previously in palm oil, was revealed. In rape oil more linolenic acid was found than had been reported before and a small amount of docosadienoic acid was shown to be present. The complete composition of rape oil in terms of component glycerides was reported to be: 54 per cent with one unsaturated C_{18} and two unsaturated C_{22} acyl groups, 28 per cent with two unsaturated C_{18} and one unsaturated C_{22} groups, and 18 per cent with one each of saturated C_{18} , unsaturated C_{18} , and unsaturated C_{22} groups. Soybean oil was found to be composed 60 per cent of monosaturated diunsaturated, and 40 per cent of triunsaturated glycerides. Two polyethenoid groups, chiefly linoleic, are present in nearly 70 per cent of the glycerides but less than 5 per cent contain three in the same molecule.

Several studies of the composition of various fats were available to the reviewers only in abstract form. Chechenkin (65) studied the highly unsaturated fatty acids of river perch by the bromination procedure, and Tsuchiya (66), in papers published several years ago, reported findings on the highly unsaturated fatty acids occurring in various marine animals, chicken legs, and the seed oil of *Oenothera lamareckiana*.

Achaya & Banerjee (67) found that the saturation is higher and the ratio of fully saturated glycerides to total saturated fatty acids is lower in Indian buffalo milk and depot fats than in the corresponding fats of western animals. Bernhard & Korrodi (68) showed that the fat of human bone marrow had almost the same composition as depot fat. Small quantities of capric, lauric, and probably decenoic and behenic acids were found in pig back fat (69). Munson *et al.* (70) isolated α -monopalmitin in a yield of 0.2 to 0.5 per cent from hog pancreas.

The finding of Boer & Jansen (71, 72) that a factor in summer butter, later shown to be vaccenic acid (73, 74, 75), increases growth in rats has stimulated interest in the occurrence of this unusual fatty acid in various fats. Bauer (76) found that beef fats contained much more (1.3 per cent) vaccenic acid than lard (0.3 per cent) and proposed the determination of vaccenic acid as a means of detecting adulteration of the latter by the former. He also called attention to the resistance of vaccenic acid to iodination because of steric hindrance. Brouwer & Jonker-Scheffener (77) reported from 4 to 5 per cent of vaccenic acid in summer butter and

1.3 to 1.8 per cent in winter butter. Geyer *et al.* (78) found considerably less (0.5 to 0.7 per cent) in butter fat of unstated origin. The difference may be related to the method of determination: Geyer *et al.* isolated vaccenic acid, while others have calculated the amount from the iodine number of the solid fatty acid fraction. Geyer *et al.* also determined the vaccenic acid content of several animal and vegetable fats and found small amounts in most of the former, and none in the latter. Groot *et al.* (79) prepared an almost pure sample of vaccenic acid from partially hydrogenated Chinese wood oil and described its properties.

The free fatty acid fraction of human hair fats was reported by Weitkamp, Smiljanic & Rothman (80) to be remarkable in two respects: first, it contained acids of every odd chain length from C_7 to C_{17} , and of every even chain length from C_8 to C_{22} ; and secondly, the double bond in the unsaturated acids was predominantly in the 6,7 position, with some ethenoid groups in the 8,9 and other positions. Normal odd carbon fatty acids have not previously been obtained from a natural source. It is unlikely that these unusual fatty acids came from the hair dressings which doubtless contaminated the sample. Indirect support for the validity of the results is given by the finding (81) that C_7 , C_9 , C_{11} , and C_{13} fatty acids possess the fungistatic action against ringworm, previously shown in the fat of adult hair (82).

Mattil & Norris (83) pointed out that truly "even" distribution of fatty acids in the triglycerides of natural fats is not proposed in Hilditch's "rule of even distribution"; on theoretical grounds no homogeneous glyceride would be expected until the molar concentration of a fatty acid exceeded 66.7 per cent, instead of approximately 60 per cent as stated in Hilditch's rule. They pointed out further that "even" distribution is not the same as "random" distribution, which is expressed by the equation

$$[S_3] = k[S]^3$$

in which $[S_3]$ is the molar concentration of triglycerides of a fatty acid, S , and $[S]$ is the molar concentration of that fatty acid. They showed by plotting data from the literature that in animal fats but not in seed or fruit coat fats, the glycerides appear to be formed by a "random" or nearly "random" distribution of fatty acid radicals among the glyceride molecules. In no class of fats is there a truly "even" distribution. These deductions were supported (84) by the application of enforced interesterification (heat-

ing at 225° for one and a half hours with stannous hydroxide as a catalyst) to animal and seed fats. The properties of the former were little affected while those of the latter were markedly altered by the treatment, a result which indicates that there was "random" distribution in the animal but not in the seed fats.

This new technique for studying the composition of fats is based on evidence discussed in the previous review (85) that when mixtures of triglycerides are heated in the presence of a catalyst, a free exchange and redistribution of component fatty acids occurs. Naudet & Desnuelle (86) added to their previous findings: they heated a mixture of equal parts of distearoolein and dioleostearin for five hours at 205° in the presence of 1 per cent sodium methylate. The product contained tristearin and triolein in amounts close to those expected if random distribution had occurred. The French workers presented several curves showing that the theoretical composition, calculated on the assumption of random distribution of fatty acid radicals, was approached when different mixtures of triglycerides were treated as described.

Chemistry of fatty acids.—Boyer, Ballou & Luck (87) continued their investigation of the combination of fatty acids with serum albumin, using an ultrafiltration procedure as a means of measuring quantitatively the amount of combination. Principal findings were that the combination between caprylate and albumin may be expressed roughly by a simple mass action relation, that it is readily reversible, and that the degree of combination increases markedly with increasing chain length from butyrate to caprate. The finding that serum albumin reduces the hemolytic action of caprylate solutions not only provided qualitative evidence for the fatty acid-protein combination but may have practical value. Further evidence that studies of fatty acid-protein combinations, such as those of Luck and his group, are of more than theoretical significance was provided by Davis & Dubos (88), who were stimulated to investigate the combination between oleic acid and serum albumin by the incidental observation that small amounts of free oleic acid, present as an impurity in the detergent, Tween 80, inhibited the growth of tubercle bacilli unless albumin was present. As measures of the binding capacity of albumin for oleic acid they employed three tests: (a) the protective action of albumin just mentioned; (b) the protective action of albumin against hemolysis of red cells by oleic acid; and (c) the effect of albumin on the opalescence of sodium oleate solutions. The results indicated that

roughly about nine moles of oleic acid are bound by one mole of serum albumin. Globulin, protamine, gelatin, and crystalline ovalbumin had no effect on the opalescence of sodium oleate solutions. Davis & Dubos believe that the binding of fatty acids by serum albumin is of much importance in nature. Fatty acid-protein compounds were prepared by Gordon *et al.* (89) in a reaction of fatty acid chlorides with proteins dissolved in alkaline solutions. Casein with approximately 20 per cent of substituent groups, ranging from caprylyl to stearyl, was made in this way.

Prout *et al.* (90) synthesized tuberculostearic acid by a series of steps which establish its structure as *l*-10-methyl octadecanoic acid. β -Linoleic acid, prepared through the tetrabromide by the procedure of Rollett (91), was shown chromatographically by Kummerow & Green (92) not to be homogeneous. It contained a small amount of yellow, viscous material which appeared to be monobromooleic acid, and which is partly responsible for the different characteristics of α - and β -linoleic acids.

Procedures for the preparation of various hydroxylated fatty acids and esters (93, 94, 95), of pure oleic acid through the hydroxamic derivative (96), and of α -, β -, γ -, and δ -chloro fatty acid esters (97) were described. Lauric acid containing C^{14} in the chain was synthesized (98). The yield of alcohols produced by the sodium reduction of fatty acid esters was greatly increased by the use of an inert solvent and the avoidance of any excess of sodium and alcohol above the amounts required in the reaction (99). The kinetics of the catalytic hydrogenation of oleic acid were studied (100). Chlorides of longer chain saturated fatty acids were found to be hydrolyzed in water at a more rapid rate than those of shorter chain acids, or of unsaturated acid chlorides of the same chain length (101). Titration characteristics of pure acetic, lauric, and myristic acids were determined (102). The solubilities of even chain, saturated fatty acids from C_8 to C_{18} in several organic solvents (103) and of a number of heavy metal soaps in chloroform and propylene glycol were reported (104).

Chemistry of glycerides and glycerol ethers.—Ferguson & Lutton (105) provided x-ray diffraction and thermal data which confirm the finding of Wheeler *et al.* (106) that triolein has three polymorphic forms melting at approximately -32° , -13° , and 5.5° . It is of interest that the same three structure types are present in the triunsaturated glyceride, triolein, as in the saturated series, tristearin, tripalmitin, etc. A similar conclusion was reached by

Carter & Malkin (107) who subjected the α -mono- and α, α' -diglycerides of erucic, brassidic, oleic, and elaidic acids to x-ray and thermal examination. The unsaturated compounds exhibited the same type of polymorphism found previously in the corresponding saturated compounds. A difference in structure was found between *trans*- and *cis*-compounds: in the former the ethenoid bond causes no appreciable change in the alignment of the hydrocarbon chain, while the *cis*-bond brings about a decided change. Charbonnet & Singleton (108) measured the heat capacity, heats of fusion and transition, and entropy of several homogeneous, saturated triglycerides.

Baer & Fischer (109) found that elaidinization had taken place during their synthesis (110) of D- α -oleyl glycerol ether. A new synthetic procedure gave a product identical with natural selachyl alcohol.

Autoxidation.—The spontaneous oxidation of unsaturated fats and fatty acids by atmospheric oxygen is of much theoretical interest and practical significance. Despite a large volume of investigative work its complex mechanism is still not clearly understood. In this limited review it is possible to give only a brief and superficial discussion of the several contributions to this subject published during the year.

Swift *et al.* (111) partially oxidized methyl oleate with oxygen at about 35° while the sample was irradiated with ultraviolet light. From the products they isolated methyl hydroperoxido oleate, about 85 to 90 per cent pure, and characterized it in several ways as being in all probability a mixture of 8- and 11-hydroperoxido octadecenoic esters. This result provides further support for the important hypothesis of Farmer & Sutton (112) that the primary product of autoxidation of unconjugated olefinic compounds is a hydroperoxide formed by the addition of a molecule of oxygen to a carbon atom adjacent to the double bond. Gunstone & Hilditch (113) found that the presence of 1 per cent methyl linoleate reduced the induction period and strongly accelerated the autoxidation of methyl oleate. The finding was explained as a catalytic action of hydroperoxides resulting from the autoxidation of methyl linoleate. Gunstone & Hilditch proposed a somewhat different mechanism for the formation of hydroperoxides: a direct addition of oxygen to the double bond to form an unstable cyclic peroxide, which rearranges to give a compound with the hydroperoxido radical on one of the carbons of the original ethenoid

group and with a new double bond in an adjacent position.

Bergström (114, 115) shook methyl linoleate in oxygen at 37° until 0.3 mole of oxygen per mole of ester had been absorbed. From the product two types of peroxide, "a" and "b", and a ketonic fraction were obtained by chromatographic separation on alumina. Fraction "a" gave a strong ultraviolet absorption, showing the presence of conjugated double bonds, and from its hydrogenated products 9-hydroxy- and 13-hydroxystearic acids were isolated. Fraction "b," which had a negligible ultraviolet absorption, yielded a mixture of dihydroxystearic acids on hydrogenation. The same products were obtained when the oxidized product was first hydrogenated and then subjected to chromatographic separation. The ketonic fraction was said to be formed during the separation. The results are in accord with the hypothesis of Farmer *et al.* (116, 117) that during autoxidation of linoleic acid a displacement of double bonds may occur through a free radical mechanism after formation of the 11-hydroperoxide. The enzymic oxidation of sodium linoleate by lipoxidase followed the same course as the autoxidation of methyl linoleate, and the same hydroxy-acids were obtained in comparable yield from the hydrogenated product (118). A small amount of a doubly unsaturated ketone was formed, apparently by dehydration of an hydroperoxide group. In a similar study Lundberg & Chipault (119) measured oxygen uptake, peroxide formation, and ultraviolet absorption during autoxidation of methyl linoleate at 40°, 60°, 80°, and 100°. Most of the absorbed oxygen was present as peroxide, but a small fraction which increased with temperature was probably ketonic. At all temperatures a constant fraction (about 70 per cent) of the peroxides was present as conjugated dienes.

Fisher *et al.* (120) in a study of partially hydrogenated oils found that the reciprocal of keeping quality, as measured by the active oxygen method, varied in proportion to the concentration of the most unsaturated fatty acid remaining in the oil. From determinations of the rates of autoxidation of a considerable number of unsaturated fatty acids and esters, Holman & Elmer (121) concluded that an increase in the number of double bonds in a fatty acid by one increases the rate of oxidation by a factor of at least two. Papers by Japanese workers on the autoxidation of unsaturated lipids are listed for reference (122, 123, 124).

Antioxidants.—Since an excellent, critical review of this subject was contributed by Mattill last year (125) only brief reference to

recent work on the antioxidants is given here. Bolland & ten Have (126) concluded from kinetic data that hydroquinone interferes in the oxidation chains in one way only, namely, by interacting with peroxide radicals; they presented kinetic evidence showing that hydroquinone undergoes chemical changes probably to benzoquinone, during this "termination" reaction. Further evidence for the degradation of antioxidants in oxidizing fats was provided by Lundberg *et al.* (127) who published kinetic data on the rates of disappearance of hydroquinone, catechol, nordihydroguaiaric acid, and gallic acid in lard heated at 100°.

The synergic effect of phosphoric acid and phenolic antioxidants [cf. (125)] was studied by Calkins (128) who found that a concentration of quinone (0.02 per cent), which had no effect by itself, inhibited the oxidation of ethyl esters of lard fatty acids almost completely if a small quantity of 85 per cent phosphoric acid was also present. Phosphoric acid was only 0.0002 per cent soluble in the esters and in that concentration it had no synergic effect with quinone. To explain these results Calkins postulated that a soluble phosphorylated quinone is formed and that the phosphoric acid, thus brought into the field of reaction, inactivates the groups which have been activated in the first stage of autoxidation.

Quantitative methods.—The low temperature crystallization procedure, developed by Brown and his colleagues (61), is proving to be a valuable tool in the analysis of natural fats. Hilditch *et al.* (62) found that it gave more precise data for unsaturated fatty acids than the lead salt separation method, though it tended to give low values for stearic and palmitic acids when they were present in high concentration. Hilditch *et al.* (64) predicted that the more informative and less tedious fractional crystallization technique would largely replace the partial hydrogenation procedure. Luddy & Riemenschneider (129) found crystallization conditions for the almost quantitative precipitation of trisaturated glycerides from lard, hydrogenated lard, and tallow. The method gave results in good agreement with the acetone-permanganate oxidation procedure of Hilditch & Lea (130) and was much less time-consuming. The periodic acid method of Pohle *et al.* (131) was modified by Handschumaker & Linteris (132) so it could be applied to the routine determination of monoglycerides in blended fats and oils.

A new technique, flowing chromatography, was applied by Claesson (133) to the separation and determination of fatty acids in a mixture. The refractive index of the solution emerging from an adsorbent filter is recorded continuously and plotted against the volume of solution. The qualitative and quantitative composition can be calculated from the characteristic curves which are obtained. The adsorption of fatty acids from nonpolar solvents on a special silica was independent of chain length, but varied with the configuration of the chain and the presence of ethenoid groups; so branched, unbranched, saturated, and unsaturated fatty acids could be separated with this adsorbent. Separation of fatty acids according to chain length was accomplished with activated carbon and polar solvents. Gracian y Tous & Pizarro (134) reported that silica was a satisfactory adsorbent for the separation of fatty acids but alumina was not.

Another new technique, counter current distribution (135), was applied to the separation of low molecular weight fatty acids (C_2 to C_8) by Sato, Barry & Craig (136). The amounts present could be estimated within 2 or 3 per cent. Weitkamp (137) used "amplified distillation" for the separation of small quantities of fatty acids. The addition of a suitable paraffin oil permitted excellent separation of methyl esters of fatty acids, but it did not work with fatty acids because of the formation of azeotropic mixtures. Ono & Toyama (138) found that the sodium salt-acetone and lithium salt-acetone methods are satisfactory for the separation of oleic from linoleic and linolenic acids. Better than 90 per cent separation was obtained. The lithium procedure was somewhat preferable.

Breusch & Ulusoy (139) isolated free fatty acids from fat mixtures by precipitation as bis-(*p*-dimethylaminophenyl) ureides. The solution containing the fatty acids in ether was boiled under reflux for two to three hours with a solution of bis-(*p*-dimethylaminophenyl)-carbodiimid. The fatty acids could be recovered from the ureides by heating with 5 per cent ethanolic potassium hydroxide. The ureides of thirty-seven fatty acids were prepared and their melting points and solubilities in several solvents were determined. Although the yield of fatty acid ureides is only about 80 per cent under the conditions described, Breusch & Ulusoy recommended that the procedure be employed in the quantitative analysis of fats.

Bloor (140) simplified his oxidative method (141) of lipid deter-

mination by measuring the change in color of the sulfuric acid-dichromate reagent with a specially designed photoelectric colorimeter.

Hills & Thiel (142), by the use of benzene-methanol as the solvent, so sensitized the ferric thiocyanate procedure as to make it capable of determining the peroxide value of freshly drawn milk. With this method autoxidation can be followed in its early stages before it is evident to the senses. Lea (143) found that in the iodometric procedure for peroxide determination it is essential to de-aerate the reagent. This was accomplished in a flask with a side tube containing the fat. Volz & Gortner (144) carried out a systematic comparison of various methods for iodometric peroxide determination and found the Kokatnur-Jelling (145) procedure of microtitration in isopropanol, as modified by Lingenfelter (146), to be preferable. Grant & Lips (147) applied various chemical determinations to rancid lards and concluded that the α -dicarbonyl test is the best chemical method for assessing rancidity. Helrich & Rieman (148) simplified the Roberts-Schuette (149) method for determining acetyl number.

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS¹

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In order to eliminate the confusion in the nomenclature of the natural amino acids and of the compounds and derivatives of these acids, a group of American chemists under the leadership of Vickery have worked out new rules for the nomenclature of natural amino acids and related substances. During the work of the committee, British chemists and carbohydrate chemists were consulted. Since the middle of 1947 the new rules have been adopted in the English speaking world (1, 2). The new nomenclature has also been used in this review.

For the proteins no new rules have been agreed upon since the "Joint Recommendations of the Physiological and Biochemical Committees on Protein Nomenclature" was published forty years ago (3). Some of the inconsistencies in this old classification of albumins and globulins have been pointed out by Jacobsen, who proposes to define globulins as unconjugated, globular proteins which can form two-phase systems with aqueous solutions at the isoelectric point, and albumins as unconjugated, globular proteins which cannot form such systems (4).

Regarding the plasma proteins the confusion in the nomenclature is most pronounced. A number of different systems has been used in order to designate the various protein fractions obtained by the numerous methods of preparation based on differences in solubility which were worked out during the last fifty years [(5) pp. 52-57]. Then ten years ago, Tiselius introduced a new nomenclature, viz., α -, β -, and γ -globulin for the serum globulins based on their different electrophoretic mobilities at pH values near 8 (6). Refinements in the electrophoresis technique soon made it evident that the α -, β -, and γ -globulins represented groups of protein with mobilities close together. The imposing development in plasma fractionation during and after the war has also resulted in the isolation and characterization of a number of new plasma proteins (7). The individual molecular constants of two α -globulins,

¹ This review covers the period from January, 1947 to December, 1947

five β -globulins and two γ -globulins from human plasma have been reported (8), but it may be expected that this is only the beginning. The time may soon be ripe to give all these newly isolated proteins individual and adequate names.

AMINO ACIDS

A very comprehensive review is given by Dunn & Rockland (9) on the synthesis, preparation and isolation of the various natural amino acids and those containing isotopic atoms. They also give a survey of the criteria of purity for the amino acids. New syntheses of DL-lysine (10), of djenkolic acid (11), of DL-aspartic acid (12), of DL-serine (13) and of methionine and similar amino acids (14) have been described. Gagnon *et al.* (15) have synthesized DL-norleucine, DL-isoleucine and DL-methionine from the substituted cyanoacetic esters. Ethyl formylaminomalonate may be used as an intermediate in the synthesis of amino acids; thus Galat (16) has prepared DL-aspartic acid by this method. By treating casein with various amounts of iodine Roche *et al.* (17) could produce moniodotyrosine, diiodotyrosine and thyroxine. An iodine-containing cysteine derivative was prepared by Jacobsen (18), and selenium analogues of DL-cystine and cysteine derivatives and of DL-methionine and DL-homocystine by Klosterman & Painter (19, 20). Certain α -amino acid esters were synthesized from malonic ester by Shivers & Hauser (21).

Vogler & Hunziker have made use of the different solubilities in water of salts of 4-nitro-4'-methyldiphenylamine-4-sulfonic acid with DL-methionine and L-methionine, respectively, for separating DL-methionine from L-methionine in partly racemized methionine (22).

The preparation of three phosphorylated amino acids was reported by Winnick & Scott (23), who treated glycine, alanine and glutamic acids with phosphorus oxychloride in aqueous magnesia solution. Histidine reacts similarly (24).

The significant problem of transamination and the function of the dicarboxylic acids in nitrogen metabolism has been reviewed by Braunshtein (25). New evidence for the important position of the aminodicarboxylic acids, primarily glutamic acid, in the synthesis of amino acids and proteins has come from Virtanen's laboratory, where Roine has studied the formation of primary amino acids in protein synthesis in yeast (26).

McIntire has prepared Schiff bases in crystalline form from free amino acids and *o*-hydroxy aromatic aldehydes (27). As the compounds formed are stable, although they may easily be hydrolyzed, their use as amino group reagents in biochemical isolation procedures has been proposed. Further evidence is reported for the configuration of methionine derived from proteins as being L-methionine (28, 29).

Methods for the determination of amino acids.—A manometric method has been developed by Braunshtein *et al.* (30) for the determination of aspartic acid. By treatment with methylsulfate and alkali, aspartic acid is converted into fumaric acid, which is reduced with zinc and phosphoric acid. The resulting succinic acid is determined in the Warburg apparatus with the aid of succinic dehydrogenase. The values obtained for the aspartic acid content of casein compare fairly well with those from Chibnall's laboratory (31) and from microbiological assay (32). The method gives too high values in the presence of asparagine, since variable fractions of this are converted to fumaric acid on methylation.

In the Graff modification (33) of the Vickery-White method for determination of cystine, cuprous oxide is used to precipitate the cystine from reduced protein hydrolyzates. As, however, under the same conditions, purines will also be precipitated, too high values for "cystine-N" may be found by the method of Graff *et al.* This uncertainty may be eliminated, however, by determining the cystine sulfur (34).

Different modifications of the colorimetric methods for tryptophane determination were reported (35, 36). These methods, making use of the glyoxylic reaction and the *p*-dimethylamino benzaldehyde reaction, cannot be used in their present form when indole or its derivatives is present (37). If, however, the solution to be investigated is made 0.5 *N* in respect to sodium hydroxide and is extracted with chloroform, the aqueous solution will contain all the tryptophane, while the indole derivatives will be in the chloroform layer. In the aqueous solution tryptophane may then be determined by means of the *p*-dimethylamino benzaldehyde.

Roche & Michel have described new micromethods for assaying monoiodotyrosine, diiodotyrosine and thyroxine (38). The method has been used to study the formation of thyroxine and its precursors in the iodinated proteins (17).

Desnuelle & Antonin have described a new method for the

quantitative determination of hydroxylysine in proteins (39). It is based on the separation of hydroxylysine from serine by ionophoresis and colorimetric microdetermination of the formaldehyde formed by the oxidation of hydroxylysine with periodic acid. These authors have also confirmed Van Slyke's observations on the presence of this natural amino acid in gelatin.

A new method for investigation of monoamino monocarboxylic acids in protein hydrolyzates has been developed by Renard (40). By treating the amino acids with a mixture of concentrated hydrochloric and nitric acids at 70° deamination takes place and the corresponding α -chloro acids are formed. The chloro acids are extracted with ether, fractionated by distillation and identified by their Raman spectra. The method has been used to show the presence of isoleucine in zein (41) and of glycine in egg albumin (42).

Virtanen & Rautanen report another method for the determination of a certain group of amino acids (43). When α -alanine, valine, leucine, isoleucine, phenylalanine and methionine are oxidized with ninhydrin, these amino acids are all quantitatively transformed to "volatile aldehydes" [$R \cdot CH(NH_2) \cdot COOH \rightarrow R \cdot CHO$] that may easily be removed by distillation. The other natural amino acids do not form volatile aldehydes.

The adsorption method, as developed by Tiselius, offers many advantages in the separation and analysis of mixtures of amino acids (44). A new and promising method for the separation of the natural amino acids into four groups has been reported by Tiselius *et al.* (45). If the mixture, dissolved in 5 per cent acetic acid, is passed through an active charcoal filter, this will adsorb only the aromatic amino acids. From the filtrate, the basic amino acids are selectively adsorbed on Wofatit C, whereas the remaining amino acids are all adsorbed on the third filter packed with Wofatit KS. After elution from the filter this group of acids may be separated by passing through an Amberlite IR-4 filter which adsorbs the acid amino acids, but lets through the neutral ones. The method has the advantage that the amino acids are obtained pure and in a salt-free condition; there is, however, some uncertainty regarding the behavior of cysteine and cystine. In an attempt to isolate lysine-rich fractions from various protein hydrolyzates, Block (46) has used a number of different cation exchange resins to pre-

pare concentrates of arginine, histidine, and lysine from such hydrolyzates. Drake (47) reports the construction of a new apparatus for recording eluate concentrations in adsorption analysis by chemical means. The apparatus was able to give a nice separation between aspartic and glutamic acids in flowing chromatography using Amberlite IR-4 as adsorbent.

The microbiological methods are playing an ever increasing role in the determination of the various amino acids, but there still seem to be new experimental factors which must be taken into consideration. It has thus been shown by Dunn *et al.* that *Lactobacillus arabinosus* 17-5, which has been used frequently for estimation of glutamic acid, is unfitted for this purpose, since D- and L-glutamic acid show different activity against this organism (48). Ågren has reported that *Streptococcus faecalis*, *Lactobacillus delbrückii* LD 5, and *Lactobacillus casei* are capable of using leucine and valine supplied in peptide form to the basal medium only when they are incorporated in certain sequences in the peptides (49). His results stress the importance of control of the progress of the protein hydrolysis, but also indicate that the findings may be further developed to identify the order in which the amino acids of peptides are linked together. According to Lyman *et al.* vitamin B₆ (pyridoxine) and carbon dioxide are important factors which modify the amino acid requirements of some of the lactic acid bacteria (50). Horn *et al.* have studied the content of several amino acids in protein and food. *Streptococcus faecalis* 9790 was used for valine (51) and threonine (52), whereas *Leuconostoc mesenteroides* P-60 was used for lysine (53). Shankman *et al.* reported a glycine content of 1.9 per cent in casein and 43.6 per cent in silk fibroin when *L. mesenteroides* P-60 was used for the assay (54). The amino acid requirements of twenty-three lactic acid bacteria belonging to the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus* were studied by Dunn *et al.* (55).

PEPTIDES

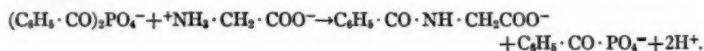
Some very interesting polymerization reactions yielding polypeptides have been reported. Katchalski *et al.* report the formation of a poly-lysine from ϵ -carbobenzoxy- α -carboxyl-L-lysine anhydride. If this substance is dried *in vacuo*, it undergoes at 105° melting and rapid polymerization, yielding polycarbobenzoxy-lysine

(56). The substance is water soluble and may serve as a model in protein research; it is split by trypsin. A macromolecular polypeptide of the structure



with very large values of n has been synthesized by Woodward & Schramm by copolymerization of N-carboxy-L-leucine anhydride with N-carboxy-DL-phenylalanine anhydride in ordinary reagent benzene at room temperature (57). The synthesis of L-leucylglycylglycine has been described (58).

Cohen & McGilvery have made an extensive study of the factors that influence the formation of *p*-amino-hippuric acid in liver slices and homogenates (59). The peptide synthesis *in vivo* has been studied by Friedberg *et al.* using carboxyl-labeled radioactive glycine (60). A very interesting finding has been made by Chantrenne, who showed that one of the anhydride groups of dibenzoylphosphate reacts very rapidly with glycine to form hippuric acid when the two substances are dissolved in a phosphate buffer at pH 7.4 and incubated at 37°C. (61). The reaction can be written:



This result demonstrates that a peptide linkage can easily be formed by spontaneous nonenzymic reaction, from an amino acid and a diacylphosphate, under physiological conditions (pH and temperature). Chantrenne suggests, as a working hypothesis, that diacylphosphates rather than monoacylphosphates could be the intermediary activated state of the carboxyl groups in the peptide-bond synthesis.

Lederer *et al.* have developed new chromatographic methods for the isolation of neutral di- and tripeptides by making use of their adsorption on acid-activated alumina from a 10 per cent formaldehyde solution (62). The glycyl peptides can afterwards be separated from the alanyl peptides, leucyl peptides, etc., as the glycyl peptides are adsorbed on acid-activated alumina from 1 per cent formol solutions, while the other neutral peptides are not (63, 64). Aschaffenburg has reported the isolation of a surface-active substance from the proteose-peptone fraction of milk (65). The substance, which so far is called σ -proteose, shows one main and two minor peaks in electrophoresis. Examined in the

ultracentrifuge it showed two peaks with $s_{20}=0.96S$ and $2.75S$, respectively (66). A crystalline, high-molecular-weight compound, probably a polypeptide, has been isolated from commercial pepsin and given the name peptophan by Bourdillon (67). Peptophan is pepsin-resistant and probably not attacked by commercial trypsin. It is not antigenic.

Penicillin is reviewed elsewhere in this volume (68).

PROTEINS

The purity of the proteins.—As the methods for investigating protein composition and structure become more advanced, the purity of the protein to be studied often turns out to be the crucial point in the whole investigation. Much too often very little attention is devoted to this important problem. The scientist is in many cases satisfied with the facts that the substance he is going to investigate is in the form of beautiful crystals and that it is prepared in almost the same way as when it was first prepared, and its purity tested according to the methods prevailing at that time. It is overlooked that the criteria for purity may have increased in the meantime, and that it may have been sufficiently pure for the type of investigation it was used for earlier. It is, of course, an æsthetic pleasure to get beautiful protein crystals, and for many investigations it is also necessary or at least an advantage to have the protein in this form. It may be dangerous, however, as the crystals may give an impression of purity which is not real. To cite Pirie (69)

Of the various criteria of purity that are used and accepted in the study of large molecules, crystallinity is the most misleading. . . . Ten recrystallizations have no bearing on a protein's purity if nothing has been removed by the last eight of them.

The demand for purity is different for the various methods used in the study of proteins. In some methods the experiments themselves may already show whether the substance itself is pure as regards a single criterion (sedimentation, electrophoresis, solubility) and if so, impurity according to some other method will usually give rise to only small errors. Thus an impurity showing the same migration as the main component either in sedimentation or in electrophoresis and amounting to 20 per cent of the substance may only change the calculated molecular weight or electrophoretic mobility, respectively, by a minute amount. In x-ray analysis

the same impurity may give rise to completely erroneous results when used for the estimation of the molecular weight and dimension of the main component. Similarly the minimum molecular weight calculated from the amino acid composition found may be wrong. In the same way the number of terminal amino groups determined according to Sanger may give false values (70). If these kinds of measurements are to be relied upon for the determination of the molecular weight of the proteins or of the number of subunits per protein molecule, great care must be taken in selecting the proper substances. It seems desirable, in such cases, that a part of the very sample of protein to be studied be first submitted to the most rigorous control for purity by as many independent methods as possible before the important investigation is started.

For the moment it seems as if the most satisfactory criteria we have for estimating the degree of purity are derived from a combination of electrophoretic analysis, the sedimentation diagrams from the ultracentrifuge, and the solubility curves in suitable solvents (71). In carrying out the tests, it is necessary, however, to analyze the electrophoretic pattern at several, sufficiently different pH values within the pH stability range of the protein. Homogeneity at a single pH value is no criterion for purity. The sedimentation analysis should be carried out with different concentrations of the protein.

The solubility tests can be classified in three different groups (72). The first may be called "Constant solvent solubility test"; it has been worked out by Northrop & Kunitz and their collaborators (73, 74, 75). The second, which may be called "Variable solvent solubility test" has recently reached a high perfection in the works of Derrien (76, 77). The third, called "Specific property solubility test," has just been introduced by Falconer & Taylor (72). It seems to be a very promising method for following the purification of any protein possessing a specific measurable property (enzymic activity, etc.) and ascertaining its ultimate purity. In all the three tests, the temperature and pH are generally kept constant. In the first method the solubility of the substance must remain the same when the composition of the solvent is kept constant. In the second method the amount of protein and the volume is kept constant while the salt concentration is increased. By plotting the amount of protein in solution (S) vs. the salt concentration (C), the precipitation of a new component will manifest itself by an

abrupt change in the slope of the S vs. C curve or a peak in the dS/dC vs. C curve. For a pure substance $\log S$ vs. C should give a straight line [Cohn (78)]. Finally the third method is carried out in the same way as the second, but the protein solubility (S) as well as the remaining specific activity (A) in the solution are determined. An analysis of the diagrams: S vs. C , A vs. C and S vs. A will give valuable information regarding the purity of the protein or indicate the experimental conditions where the chances of success are best for a further purification. For a pure substance A must be of course directly proportional to C .

Structure.—Much valuable information about the structure of the largest protein molecules, the viruses, has been gained already by their investigation in the electron microscope. The instrument is still in development and we may expect that it will be of great value in the elucidation of the gross structure of protein molecules other than viruses. Already some remarkable achievements have been made, however, in Wyckoff's laboratory where the hemocyanin molecules from *Busycon canaliculatum* have been studied (79). Electron micrographs of sufficiently diluted hemocyanin near its natural pH showed that each particle consists of rod-like subunits stacked together in bundles of four. The subunits are about three times as long as they are thick and have pairs, or all four, of their long axes parallel giving a cube-like particle of constant dimensions. At a few places one pair of rods seems to be rotated 90° with respect to the other, so that such a tetrad shows the ends of two rods and the side view of another. At higher pH values the elongated subunits are observed, but above pH 10 they are disintegrated. Even the comparatively small molecules of γ -globulin have recently been discerned (80). Beautiful electron micrographs showing the molecular particle arrangement have also been obtained from concentrated solutions of tobacco mosaic virus protein, from films dried from such solutions (81), and from tobacco necrosis virus crystals (82).

The x-ray study of protein structure is reviewed elsewhere in this volume (83) and will not be treated here. A very important paper just published from the Cavendish laboratory by Boyes-Watson, Davidson & Perutz must, however, be mentioned (84). From a very careful analysis of the x-ray diffraction pattern from single crystals of horse methemoglobin under various experimental conditions, these authors conclude that the molecules of this pro-

tein resemble cylinders of an average height of 34 Å and a diameter of 57 Å. Each molecule of mol. wt. 66,700 must consist of two chemically and structurally identical halves; furthermore each molecule contains four layers with prominent concentration of scattering matter just under 9 Å apart and parallel to the base of the cylinder. The analysis of the diagrams also indicates that interatomic vectors of 9 to 11 Å occur frequently in many directions. The authors give no further structural interpretation of their results, but there seems to be considerable resemblance between their results and the hypothesis put forward by Dervichian for the structure of some of the protein molecules based on measurements on protein films (85). This might also explain why horse hemoglobin is so easily reversibly dissociated to half molecules, but not to fourth molecules.

In the wet monoclinic hemoglobin crystals the unit cell contains two molecules and 52.4 per cent of liquid of crystallization, the latter consisting of two distinct components: water "bound" to the protein and not available as solvent to diffusing ions, and "free" water in dynamic equilibrium with the suspension medium. It was found that the liquid of crystallization could be varied by swelling and shrinkage of the crystals in suitable solution. This produced stepwise, reversible transitions between different well-defined lattices. The protein molecules were not involved in these changes, and two of the dimensions of the unit cells remained almost unchanged (84, 86). As soon as the "bound" water was removed considerable changes appeared in the regularity of the x-ray pattern, and the reflections corresponding to all the short spacings disappeared. A difference between two types of liquid of crystallization had been found earlier by McMeekin & Warner (87, 88). From a small angle x-ray study of the red corpuscles, Dervichian *et al.* conclude that the hemoglobin molecules inside the corpuscles must be arranged in some kind of pattern (89).

Fractionation of protein solutions.—During the war and after, great progress has been made in the fractionation of protein solutions and in the isolation of individual components. The imposing development of the alcohol fractionation technique has already been reviewed from different points of view in several places (7, 90, 91, 92). As an example, it may be mentioned that more than thirty protein components have been separated and concentrated from human plasma (93).

After the conclusion of last year's review (90) some important papers dealing with the plasma fractionation have been published. Deutsch *et al.* have made new fractionations of the Fraction II + III from human plasma resulting in electrophoretically comparatively pure γ_1 - and γ_2 -globulins (94). The name γ_1 -globulin was earlier introduced by these authors (95) to designate the electrophoretic fractions between β - and γ -globulin, by some authors called β_2 -globulin. It corresponds to the "T-component" of Van der Scheer *et al.* (96). Neither the γ_1 - nor the γ_2 -globulin was homogeneous in the ultracentrifuge, and marked differences in the antibody activity were found in the γ_1 - and γ_2 -globulins; thus the hemagglutinins and typhoid "O" agglutinin appeared to be γ_1 -globulins. Part of the $s_{20} = 20$ S component from γ_1 consisted of the β -hemagglutinins, in agreement with earlier findings (5).

The isolation and crystallization of serum albumins from ethanol-water mixtures have been reported from the Harvard laboratory (97). The difficulty in getting the serum albumins to crystallize has been overcome by the addition of small amounts of some crystallization aids. The most effective was *n*-decanol, but a number of other substances were also active, as for instance aliphatic alcohols containing five or more carbon atoms, *n*-amyl chloride, benzene, toluene, chloroform and ethylene dichloride. Hughes has reported that human serum albumin can also be crystallized as a mercury salt (98). The salt is only sparingly soluble in water, but dissolves readily in 0.2 *M* NaCl. Analytical evidence suggested that two albumin molecules are linked together through one atom of mercury. This hypothesis is strongly supported by the finding that freshly prepared neutral solutions of the crystals revealed two peaks in the ultracentrifuge; a main component with $s_{20} = 6.5$ S and a small amount with $s_{20} = 4.6$ S, the normal value for serum albumin. Upon standing the amount of the dimer decreased. Some recent ultracentrifugal observations made in Upsala indicate that the crystallization aids mentioned above (97) may also produce a dimer formation. Thus Bresler has found that aqueous solutions of bovine serum albumin show varying amounts of a second peak with $s_{20} \sim 6.5$ S after the addition of benzene, ethyl ether and several other substances (99).

From the fibrinogen-rich Fraction I of human plasma, Edsall *et al.* have isolated an asymmetrical, nonclotting protein (100) having approximately the same length as fibrinogen, but twice the

cross-sectional area (101). It is suggested that this protein could be a dimer of fibrinogen unable to take part in fibrin formation. A new and remarkably simple method of preparing very pure fibrinogen has been reported from Seegers' laboratory (102). The fibrinogen was precipitated by freezing and thawing, and subsequently washed. The range of purity is from 95 to 100 per cent, and the fibrinogen obtained has many qualities not possessed by former preparations. Thus the tensile strength is directly proportional to the fibrinogen concentration over a wide range, and it is increased by calcium ions.

Smith & Gerlough have tried to isolate the tetanus antitoxic activity of hyperimmune horse plasma by means of the alcohol fractionation technique, but they found that it was present in at least three distinct proteins: T-globulin, γ -globulin, and a β -pseudo globulin (103). A similar widespread occurrence of the type-I antipneumococcus activity in various globulin fractions has been reported earlier by Grönwall (104).

The ethanol fractionation procedure of the Harvard group has been modified in Chanutin's laboratory in order to study the changes produced in the serum from rats after thermal injury (105) and from dogs injured by turpentine, heat, and bis(β -chloroethyl) sulfide (106). The last paper contains very detailed fractionation schemes for dog serum and gives much interesting information regarding the changes occurring in the α - and β -globulins following the various injuries.

Cohn & Koechlin have reported the isolation and crystallization of a metal-combining β_1 -pseudoglobulin from the fraction IV-7 of human plasma by the ethanol procedure (93, 107). The same protein had been independently isolated and characterized by Laurell *et al.* by a combination of ammonium sulfate fractionation and ethanol fractionation at low temperature (108, 109).

Various modifications of the old method of salt fractionation have been used to isolate certain proteins. Wunderly describes experiments on the preparation of γ -globulins from various animals using diverse methods (110). The purity of the γ -globulin he obtains in the first fractionation, corresponding to 0.33 saturated ammonium sulfate, is rather poor. A simple dilution of the sera before the salting-out or a few washings of the precipitate would have resulted in a much purer starting material [(5) pp. 63-67].

Theorell & de Duve have isolated and crystallized human

myoglobin both from heart muscle and from myoglobin-containing urine (111). The hemoglobin from *Gastrophilus* larvae has been purified and crystallized by Keilin & Wang (112). It differs from blood hemoglobin of the horse (host of the larvae) in many respects, thus showing that the larvae must synthesize their own hemoglobin.

Kabat *et al.* have purified ricin, the highly toxic hemagglutinating protein of the castor bean, by fractional precipitation with sodium sulfate (113). It was found to be electrophoretically, ultracentrifugally, and immunochemically 100 per cent pure, but when it was crystallized, the crystals were 50 per cent more toxic! There was an indication that castor beans contain both the toxic and the nontoxic forms.

Some new methods for preparing various enzymes have appeared during the year. It has been difficult to prepare pure liver esterase because this enzyme is very firmly bound to the insoluble cell substances. However, this property is used in a method developed by Falconer & Taylor (114) in which liver mince is first extracted a few times with water in order to remove the water soluble material. Then the pH of the suspension is changed to a suitable value and a much purer crude extract is obtained than in the previous methods. The final purification of the crude extract by salt fractionation is guided by means of the "Specific property solubility test" of the same authors (72). A detailed description of the purification of cholinesterase from the tissues of *Electrophorus electricus* by fractional ammonium sulfate precipitation has been given by Rothenberg & Nachmansohn (115).

Meyer *et al.* have succeeded in isolating α -amylase in a remarkably pure state from swine pancreas (116). The problem was a very intricate one, as the amylase is easily denatured and as the pancreas contains a large number of enzymes and other proteins. It was later crystallized from concentrated amylase solutions (117).

A crystalline lipoxidase has been prepared by Theorell *et al.* by a combination of fractionation with ammonium sulfate, precipitation with ethanol in the cold, electrophoretical isolation of the active protein, and crystallization of the enzyme with ammonium sulfate (118, 119).

The advantages of adsorption separation methods have not yet been fully utilized in the protein field. It is not always possible to find suitable adsorbents which are both powerful enough and

reversible. It is therefore of interest that Tiselius recently has shown that some adsorbents—like cellulose (filter paper) and silica gel—which normally show very small affinity for proteins, in presence of salts of moderately high concentration become very efficient adsorbents (120). Elution is made simply by treatment with water or dilute salt solutions. "Salting-out" adsorption may become of considerable value for preparative and analytical work with proteins and other substances of high and moderately high molecular weight.

An interesting new modification of the salting-out technique has been described by Astrup & Birch-Andersen (121). These authors made use of protein-precipitating agents such as sulfo-salicylic acid and tungstic acid at suitable pH values and were able to change the sequence for the precipitation of the globulin components in bovine serum. It seems as though with further study of the conditions and the nature of the agents used for formation of the slightly soluble protein compounds this method should offer certain possibilities for obtaining more selective precipitations of proteins. It appears that it is generally easier to get a selective precipitation with protein-precipitating anions than with cations. The method has been used in the purification of renin (122).

Salting-out curves.—In many cases it is of interest to know how many individual components a given protein solution contains. One way of finding out is to study the solution by means of electrophoresis and ultracentrifugation. These two methods supplement each other very well and have been greatly used during the last decade. In recent years much interest has been devoted to the study of salting-out curves as a means of studying the number of components in protein solutions and their relative amounts. Much work has also been done to correlate the results obtained from salting out with those from electrophoresis and sedimentation.

The old sodium sulfate method of Howe (123) has been used by Majoor to study the salting-out curves for normal and pathological human sera (124). When comparison was made with the electrophoresis diagrams for the same sera, it was found that euglobulin, precipitated with 18.5 per cent sodium sulfate, corresponded fairly well with γ -globulin, while the pseudoglobulin, precipitated with 26.8 per cent, was not too different from the sum of α - and β -globulin (125). Milne has reported similar experiments, but he finds that when euglobulin is precipitated with 19.6 per cent and

pseudoglobulin with 26.8 per cent, the former is equal to the sum of γ - and β -globulins, while the latter corresponds to the sum of α_1 - and α_2 -globulins (126). In the classical Howe method the globulins are precipitated, however, with 22.2 per cent sodium chloride. Under these conditions, the proteins remaining in the solution are albumins as well as α_1 - and α_2 -globulins (127).

The potassium phosphate method of Butler & Montgomery (128) has been increasingly used for the study of a number of different sera (129 to 132). A high degree of perfection in using this method has been achieved by Derrien (76, 77). On the salting-out curve from a normal human serum (pooled sample) he thought it possible to disclose the presence of thirty to thirty-five individual components. In order to be able to arrive at such conclusions, it is necessary to determine a great number of points on the salting-out curve. In his "Research technique" Derrien bases his curve on one hundred to one hundred-forty experimental determinations, while in the "Routine method" the curve is based upon forty to fifty determinations. The experimental conditions must be rigorously controlled, and sufficient time must be allowed for the solutions to reach equilibrium with the corresponding precipitates.

The interesting observation has been made by Jayle & Gillard that the salting-out curve for a single component in a protein mixture is dependent upon the way in which the salt is added (133). When the salt solution is poured into the protein solution, the precipitation zone is much broader than when the salt is allowed to diffuse through a membrane. The precipitation starts also at a lower salt concentration in the former case than in the latter.

In some recent experiments, Derrien has taken out fractions between rather narrow limits of salt concentration. These fractions have then been studied in electrophoresis and their salting-out curves have been determined. Subfractions from normal as well as pathological sera were examined and a remarkably good correlation was found between the two methods (134). The subfractions obtained between narrow limits of salt concentration are naturally not pure, since there is always some overlapping in the precipitation. The globulin components, precipitating before the albumins, come out in the following order, γ , β_2 , γ , β_1 , α_2 , β , β plus α , α_2 plus α_1 (134). This may explain why it is often difficult to get complete correlation between the results from salt precipitation and from electrophoresis.

The number of components and their relative amount found from salting-out curves may of course not always reflect the absolute composition of the native protein solution. Some compounds existing in the solution may be broken up by the high concentration. Some of the components still in solution may be increasingly adsorbed [salted-out according to Tiselius (120)] on to the precipitate already present in the solution. Apart from these inevitable handicaps that are common to all salting-out methods, Derrien's method is a valuable addition to the methods of studying protein mixtures.

Denaturation.—The role played by the masked sulfhydryl groups in the stability of the proteins and in the denaturation of these substances has been extensively studied in Desreux's laboratory (135 to 139). The reactions of these groups with various agents, especially chloropicrin and iodine, have been tested chemically as well as physicochemically before and after denaturation of the proteins.

The effect of x-rays on the hemocyanin from *Limulus polyphemus* has been studied by Pickels & Anderson who found association taking place probably to aggregates of two, three or four primary particles (140). Addition of horse serum or egg albumin before the irradiation inhibited or decreased the effect of the x-rays. An increase of the oxygen pressure in the solution acted in the same way. A very strong delayed effect has been reported after irradiation of pepsin with x-rays (141).

Prudhomme & Grabar have subjected different fractions from horse serum to ultrasonic vibrations at 960 kilocycles (142). Among the changes produced, the most remarkable was a loss of the characteristic ultraviolet absorption band at 280 $\mu\mu$. In accordance with this result, the free aromatic amino acids were found also to lose their ultraviolet absorption after ultrasonic treatment. The phenomenon occurred in the presence as well as in the absence of oxygen and seems, strangely enough, to be due to the opening of benzene rings.

Some very interesting results of treating β -lactoglobulin with urea at different temperatures have just been reported (143). Lactoglobulin solutions containing 38 per cent urea were left for different lengths of time at various temperatures and the amount of denatured lactoglobulin was determined at intervals, assuming that only native lactoglobulin was soluble in a certain acetate

buffer containing 0.5 *M* MgSO₄. Under these assumptions the denaturation of β -lactoglobulin was found to have a negative temperature coefficient, and to be reversible. At 0° and 10° the denaturation was very rapid; in three hours about 85 per cent was denatured, whereas at 37° only 70 per cent was denatured after fifteen hours. If a sample after treatment at 0° was only 30 per cent soluble in the test buffer, a short treatment at 37° was sufficient to make it 97 per cent soluble in the same buffer. The reviewer wonders whether the changes observed may not be due to change in the hydration of the lactoglobulin molecule similar to the different methemoglobin hydrates studied by Perutz *et al.* (84, 86).

PHYSICOCHEMICAL INVESTIGATIONS

The work of Svedberg on the molecular weights of proteins, which was started a little more than twenty years ago, showed for the first time that the soluble proteins, now generally called the globular proteins, have definite molecular weights in solution. Svedberg also advanced the hypothesis that the molecular weights of these proteins were simple multiples of some basic unit (17,600) (144). There is no doubt that this bold hypothesis gave a very important impetus to the whole study of the proteins and caught the interest of scientists from other fields than chemistry. The multiple hypothesis has in later years been severely criticized (145, 146). Undoubtedly the hypothesis is not of such general validity as first seemed to be so, but still it seems quite possible that we have several parallel sets of multiple systems each characterized by the specific nature, function, and process of formation of the proteins in question. The molecular weights of the respiratory proteins could probably be adequately described by two (or perhaps three) multiple systems with different basic units. Unfortunately the molecular weight has been determined for only a small fraction of all the respiratory proteins studied in the ultracentrifuge [(144) pp. 355-72]. Generally the sedimentation constant alone was measured, but these data, combined with the results from the investigations of the pH-stability diagrams and of association and dissociation reactions strongly support the hypothesis of multiple systems for this group of proteins. The reviewer is also inclined to think that when more data are collected for distinct groups of proteins, it will be found that their molecular weights may be governed by a multiple law.

Sedimentation.—Different values for the sedimentation constant of crystalline insulin have been obtained in Oxford (147) and Upsala (148), viz., 3.34 S and 3.55 S respectively. As, however, the pH stability range observed in the two laboratories was different, there is reason to believe that despite the use of crystalline insulin the two samples must have had a different protein composition. It would be of interest, however, to have a standard substance, better defined than insulin, to test the sedimentation constants obtained in the various laboratories.

There is some inconsistency in the reporting of the sedimentation constant, some laboratories give $s_{20}^{1\%}$, the sedimentation constant in a 1 per cent protein solution in pure water at 20°, while others give the sedimentation constant extrapolated to zero protein concentration in water at 20°, viz. s_{20}^0 . When it is given as s_{20} it must be assumed to refer to zero protein concentration. The sedimentation constant of the proteins (and other high molecular substances) varies considerably with their concentration when run in the centrifuge [(5) pp. 153–65]. For the calculation of the molecular weight from sedimentation velocity runs it is therefore always necessary to extrapolate the s vs. c curve to zero concentration. It has been suggested that the variation in s could be eliminated by multiplying the observed sedimentation constant (s_{obs}) by the relative viscosity of the protein solution (149, 150). The application of this correction, however, often leads to an overcompensation (150, 151).

The variation of s with c may be explained, at least to a first approximation, in a much simpler way according to Enoksson (152). What we want to determine in a run in the ultracentrifuge is the sedimentation constant (s_0) of, for instance, the protein molecules relative to the solvent. In an actual run, however, we observe s_{obs} for the protein molecules relative to the walls of the ultracentrifuge cell, but s_0 will be equal to s_{obs} at infinite dilution only. In a solution with the protein concentration c (in grams of unhydrated protein per cm^3) we get as a first approximation, assuming s_0 is independent of the protein concentration

$$s_{obs} = s_0(1 - c\Phi)$$

where Φ is the "specific hydrodynamic volume" of the sedimenting particle, i.e., the specific volume of the hydrated particles including the solvent (salt solution) they carry along while sedimenting. From

the variation of s_{obs} with the protein concentration it should thus be possible to estimate Φ . The expression for s_{obs} has some features in common with one derived earlier by Burgers (153).

It has long been known that when mixtures of two proteins with different s_{20} are studied in the centrifuge, the concentration calculated from the diagram for the rapidly sedimenting component gives too low a value, while the opposite is the case for the slower component [(144, p. 408) (154, 155)]. Many different explanations have been given for this phenomenon, but recently Johnston & Ogston described it as a physical boundary anomaly assuming that the sedimentation velocity of the slowly moving component is decreased in the presence of the faster moving one (156). It is now evident that the phenomenon is of a physical nature, but a more natural explanation is offered by the following considerations. In the original solution we have per cm^3 of solution c_1 gram of the faster moving component and c_2 gram of the slower. When the faster component disappears, the volume it occupied is taken up by solution containing only the other component. One cm^3 now contains $c_2/(1-c_1\Phi_1)$ gram of the slower component, and as the concentration of the faster component is measured relative to this concentration, which is larger than c_2 , it comes out too low (152).

In a similar way the concentration calculated for a protein sedimenting in a solution containing low molecular weight substances must be too low. From the difference between the original and the calculated protein concentrations the specific volume for the protein plus "bound water" may be computed (152). The same principle has earlier been used by Sihtola & Svedberg for determining the solvation of nitrocellulose (157). A phenomenon similar to the one just described must also be effective in electrophoresis and causes errors in addition to those discussed by Svensson (158).

Ultracentrifugal studies on the dissociation process of the hemocyanins have been reported by Brohult (159). The molecular dimensions computed for the whole, half, and eighth molecules agreed fairly well with results from stream double refraction, electron microscope, and monolayer measurements. All the results indicated that the three molecular forms had the same length. The dissociation is supposed to start with a swelling of the molecule, subsequently followed by a lengthwise splitting. It was found that the hemocyanin from *Helix pomatia* is composed of two kinds of

molecules, A and B. A, which constitutes 75 per cent, is dissociated by electrolytes as well as by changes in pH. B is dissociated only by changes in pH. A has been isolated by high speed centrifugation and by precipitation with ammonium sulfate. Another hemocyanin, from *Paludina vivipara*, was found to be dissociated by changes in pH, but associated after the addition of electrolytes.

Adair *et al.* have measured the osmotic pressure and sedimentation velocity of *Gastrophilus* methemoglobin. The osmotic pressure gave a molecular weight of 34,000. A homogeneous component with $s_{20} = 2.5$ S was found in the ultracentrifuge (160). The diffusion constant for α -amylase has been determined from electrophoresis experiments (161), but the value, $D_{20} = 12.0 - 12.5 \times 10^{-7}$, is undoubtedly too high. α -Amylase from the same laboratory in special diffusion experiments gave $D_{20} = 8.1 \times 10^{-7}$, and in the ultracentrifuge $s_{20} = 4.5$ S, giving a molecular weight of 45,000 (162).

Archibald has made an approximate solution of the differential equation for the ultracentrifuge (163). It has not yet been experimentally tested, but it should be of special value in the study of molecular weights of proteins and peptides in the region 1000 to 10,000. A new optical method for the simultaneous determination of the concentration distribution as well as the concentration gradient distribution in sedimentation equilibrium has been developed by Kegeles (164).

A very important improvement in the accuracy of the determination of the diffusion constants for pure substances seems to be possible after Longworth's introduction of an interference method for studying diffusion (165, 166) and after the introduction of a new diffusion cell (167). For measurements where the highest precision is not needed, a new cell construction (168) and a new method for forming the boundary (169) should be of some value.

Electrophoresis.—After the introduction of Tiselius' new electrophoresis technique ten years ago (170), this method has been used in ever increasing degree, and it is now considered one of the best methods for testing the purity of protein solutions. An overwhelming number of papers dealing with electrophoresis were published last year, but due to lack of space they cannot be dealt with in this review. It seems, however, that with the increased use of this method, many uncritical experiments have been performed and published. Just as valuable as this method is in the hands of the person who knows the technique and is familiar with

its many sources of error, by so much is it dangerous in the hands of persons who know only little about it.

The application of electrophoresis for biological and medical problems (171, 172) and for preparative purposes (173) has recently been reviewed. A very instructive description of the electrophoresis technique has been given by Wiedemann (174). The same author has proposed to eliminate the δ - and ϵ -boundaries in electrophoresis by increasing the salt concentration in the supernatant buffer solution (175). The reviewer must warn against this change in the experimental conditions; it is of questionable value and may easily involve the risk of convection currents.

INVESTIGATIONS ON INDIVIDUAL PROTEINS

The amino acid composition of proteins and peptides.—The amino acid composition of Kunitz' crystalline chymotrypsinogen has been given by Brand (176). Using the symbols earlier proposed by him (90, 177) the composition is given as:

Chymotrypsinogen, mol. wt. = 36,800, total N = 16.18 per cent
 Gly₂₆ Ala₁ Val₃₂ Leu₂₉ Ileu₁₆ Pro₁₉ Phe₃ (CySH)₄ (CyS-)₁₀ Met₃
 Try₁₀ Arg₆ His₃ Lys₂₀ Asp₁₃ (Glu-NH₂)₂₂ (Asp-NH₂)₁₈ Ser₄₀ Thr₃₅
 Tyr₆ (H₂O)₅

By this formula 98.3 per cent of the total nitrogen has been accounted for, but only 95.3 per cent of the protein by weight on a residue basis. According to Brand, this discrepancy indicates the possibility that chymotrypsinogen may contain a nitrogen-free or nitrogen-low constituent. The complete amino acid composition of crystalline *Clostridium botulinum* type A toxin has been reported by Buehler *et al.* (178). Nineteen amino acids were found and a minimum molecular weight of approximately 45,000 was calculated as compared with mol. wt. ~900,000 from sedimentation and diffusion (179). The extreme toxicity of this protein could not be explained by the amino acid composition as such. Another complete amino acid analysis has been reported by Tristram for the protamine salmin (180):

Salmin mol. wt. = 8,000, Arg₄₀ Ileu₁ Val₂ Pro₄ Gly₃ Ala₁ Ser₇

Knight has made a very interesting study of the variation in the amino acid composition of eight different strains of tobacco mosaic virus (181). Some striking variations among the amino acid con-

tents of various strains were found. Thus the J14D1 strain, which is supposed to have arisen from the TMV strain by two mutations, shows definite differences in composition only in respect to lysine and glutamic acid. Knight suggests that mutation may involve stepwise changes in the amino acid content of the virus.

In several investigations only certain of the amino acids from the protein hydrolysate were determined, mostly by means of microbiological methods. Thus Franklin, Li & Dunn determined thirteen amino acids in an electrophoretically pure preparation of the anterior hypophyseal growth hormone (182). On the basis of the histidine content and earlier osmotic pressure measurements by Li *et al.* (183), a molecular weight of 46,800 was estimated for the hormone. For another hormone, secretin, Edman & Ågren have reported the presence of fifteen (or sixteen) amino acids, and they have given quantitative data for seven of these (184). From the tryosine content a minimum molecular weight of 5,250 is estimated. The values for total sulfur and cystine, however, indicate a molecular weight twice as high.

Knight has determined the content of seventeen different amino acids in highly purified influenza virus particles (PR8 and Lee strains) and in the sedimentable particles of normal allantoic fluid (185). Half of the amino acids were the same in the two virus strains. The composition of particles from normal allantoic fluid resembled that of the Lee virus for eight or nine acids, but resembled that of PR8 virus for only four or five acids.

Smith *et al.* have studied the content of twelve amino acids in six different crystalline seed globulins (186, 187). It is very characteristic that these seed globulins have a high arginine content, about 16 per cent. A point of uncertainty in the analyses was that the sum of the cystine and methionine content did not completely account for the total sulfur content of these proteins. Smith *et al.* have reported new data on the amino acid composition of immune proteins and globulins from milk and colostrum (188). The new results further emphasize the conclusions already mentioned in last year's review (90). For all these proteins the sulfur-containing amino acids account reasonably well for their total sulfur content. Some amino acid analyses of a purified colostrum pseudoglobulin have been made by Hansen *et al.* (189), but their results for several of the acids do not agree well with the above mentioned (188).

Vassel *et al.* have made a comprehensive study of the changes produced in dog serum before and during type I pneumococcal pneumonia (190, 191). In the first paper determinations of a number of amino acids are reported for crystalbumin, globoglycoid, seroglycoid and "proteose," prepared according to Hewitt (192) from normal dogs and from dogs with mild and severe pneumococcal pneumonia. The experiments show some very striking changes especially in the serine and threonine content. In their second paper (191), analytical values for the same amino acids are reported on the euglobulin, and pseudoglobulins I and II fractions, but in this case the amino acid composition remained essentially the same for most of the amino acids. It seems to the reviewer a great pity nowadays to make such an extensive study without controlling the fractions obtained by means of, for instance, electrophoresis. The changes observed in the first paper would probably have manifested themselves clearly in the electrophoresis diagrams.

Muscle proteins.—At present the problems of the muscle proteins are attracting widespread interest, and this may also be one of the fields where quite revolutionary developments may occur. Due to lack of space only a few of the recent studies on these substances can be mentioned.

A very stimulating book giving the results and theories of the Szent-Györgyi school has been published (193). A group of acid- and heat-stable proteins, among them the chromoprotein fluorochrome, and a cerebroside, were shown to be constituents of crystalline myosin. These substances are involved in enzymatic reactions as well as in actomyosin formation (193). Experiments carried out with muscle slices and actomyosin threads indicate that contraction is an endothermic process. Relaxation can occur only if the free energy of the system is increased by seven thousand calories (193). There exists some similarity between the structural proteins of muscle, kidney and brain (193).

The problem of muscular contraction has been discussed before the New York Academy (194). Reviews dealing with various problems relating to the muscle proteins have been written by Engelhardt (195) and Buchthal (196).

The myosins, prepared according to Greenstein & Edsall (197), show numerous components in the ultracentrifuge while Szent-Györgyi's crystalline myosin shows only a single component with $s_{20} = 7.2S$. Combined with the result from diffusion measurements

($D_{20} = 0.5 \times 10^{-7}$) a molecular weight of 1.5×10^6 is estimated (198). Salting-out curves according to Derrien (76, 77) showed that crystalline myosin consists of four main components (198).

Electrophoretic experiments showed that crystalline myosin dissolved in potassium chloride is negatively charged at pH 7, whereas it is positively charged at the same pH when dissolved in an excess of calcium chloride. The isoelectric point of myosin dissolved in this salt is profoundly changed, and in the presence of 0.22 *M* calcium chloride it is more alkaline than pH 9 (199).

Actin, in the presence of 0.5 *M* KI, which inhibits polymerization, has a molecular weight of about 70,000 (198). Experiments with actin and actomyosin are greatly hampered by the gelling tendencies of these substances.

The extraction of actin from muscle and from actomyosin and the characteristic reversible filament formation have been confirmed by studies with the electron microscope. The filament formation appears to be a linear aggregation of the corpuscular particles into long filaments with diameters between 80 and 140 Å. The actomyosin showed "filaments which are wider than those of actin and which bear a resemblance to filaments of myosin extracted in alkaline salt solution" (200). Similar electron microscope results have also been obtained elsewhere (201). Astbury devotes a large part of his Croonian lecture to problems connected with the muscle proteins (202) and he has published a short note on x-ray studies of actin (203).

An interesting new method of protein extraction from rabbit muscles has been introduced by Amberson and co-workers (204). By this method the rabbit is first exsanguinated by perfusion with Ringer-Locke solution so as to remove practically all the blood from the muscles before these are taken from the rabbit and extracted. The muscle is then quickly frozen to $-70^{\circ}\text{C}.$, in closed vessels placed in carbon dioxide snow, after which it is extracted at 0° to $+2^{\circ}\text{C}.$ for several days (or weeks) with a suitable salt solution without preliminary mincing. If the extraction is carried out with 0.4 *M* potassium phosphate (pH 7.6) considerable amounts of a few proteins, comparatively well defined electrophoretically as well as ultracentrifugally, are extracted. If, however, sodium pyrophosphate is added to the extraction medium large amounts of myosin are extracted in addition to other components. This very gentle method of extraction, without mincing of the material,

may be of more general applicability, especially when native biological material has to be extracted. [Cf. also the view put forward by Tyler (205, 206).]

Miscellaneous.—Many interesting publications have appeared about the reactions between proteins and a number of chemical reagents. Unfortunately, space does not permit a detailed mentioning of these papers, and reference shall only be made to two recent reviews (207, 208). New studies have been reported of the reaction between serum albumin and organic ions (209), and some of these could be studied quantitatively and correlated with the law of mass action (210, 211).

Some interesting combinations between differently charged proteins have been reported by Kleczkowski (212). Morrison has studied the various factors which influence the quantitative determination of fibrinogen (213). It was found that proteins of high molecular weight and of great asymmetry as well as certain enzymes and lipoproteins were strongly occluded, while other small molecules were not.

Li has made the rather unexpected finding that crystalline β -lactoglobulin consists of two or three major components (214). His result has been confirmed in Upsala (215). There is, however, a slight possibility that enzymatic changes like the ones next described (216) may cause such changes. For a long time β -lactoglobulin was considered a prototype of a homogeneous well-defined protein and it had been used as such for numerous investigations. It does not seem unlikely that other of our "standard proteins" may suffer the same fate in the years to come.

A very interesting observation has been made at the Carlsberg laboratory, where it was found that in some old samples of egg albumin this protein had been changed to a new, much more soluble protein that could easily be crystallized (216). Investigation of the process showed that the transformation could be catalyzed by bacterial enzymes from *Bacillus subtilis*. The new protein seems to be distinctly different from egg albumin in several respects, but it has about the same molecular weight as egg albumin according to osmotic, sedimentation, and diffusion measurements (217). Similar processes of protein transformation have so far been observed only in the field of enzyme chemistry, where the transformation of an inactive zymogen leads to an active enzyme. It seems likely that similar processes could have passed by unob-

served in many cases in the past. The observation of Linderström-Lang & Ottesen may open a number of new perspectives to protein chemistry and allows us to transform one well-defined protein to another well-defined protein.

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NUCLEOPROTEINS, NUCLEIC ACIDS, AND RELATED SUBSTANCES¹

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This review article must start, unfortunately, with a tribute to the memory of J. M. Gulland. His death means a sad and irreparable loss to us all.

The field under consideration here has, in the past few years, witnessed a renaissance of interest, as signified by the large number of sometimes excessively repetitious reviews, conferences and symposia. The biological importance of nucleic acids and nucleoproteins is perhaps also indicated by the appearance of several substances which, in their names, claim descent from such distinguished ancestors as the chromosomes or even the genes. Whether some of these designations are justified, remains to be seen.

The present state of our knowledge of nucleic acid chemistry could perhaps be compared to that in which protein chemistry found itself at the beginning of this century. The main constituents are known, at least as regards the principal nucleic acids from calf thymus and from yeast; but a real insight into the order and mode of their arrangement is lacking and so is an understanding of the chemical basis of structural specificity. The powerful tools of immunochemistry, so effective in demonstrating the immense number of structurally different proteins occurring in nature, have so far been of no avail, perhaps because of the presence in the organism of depolymerizing enzymes. With the gradual recognition of the macromolecular character of many nucleic acids and the concomitant relinquishment of the tetranucleotide conception, objections to the existence of a large number of structurally different nucleic acids began to disappear, even before the discovery of bacterial transformation by specific desoxypentose nucleic acids. In the absence, however, of immunological or more precise chemical methods of general applicability it is as difficult to prove as to disprove the identity of nucleic acids synthesized in different cellular systems; and where the identification procedures customary in

¹ This review covers the period from 1945 to approximately November, 1947.

organic chemistry fail, especially with compounds of very high molecular weight, the entire conception of chemical identity becomes meaningless.

It is customary, and this article will adhere to the convention, to distinguish between two main groups of nucleic acids, the desoxypentose and pentose nucleic acids.² But it is by no means certain that this difference in the sugar components is the most important demarcating feature (one may as well attempt to distinguish between thymine and uracil nucleic acids); and since the nucleic acid molecules of the same sugar type may not all have the same biological function in the cell, we may actually in many cases, even in an ostensibly homogeneous preparation, be dealing with a mixture of a large number of chemical entities.

The preceding survey of the field, published in this *Review* three years ago (1), has summarized our present knowledge of the structure of nucleic acids so excellently that a repetition of this approach appears superfluous. The discussion that is to follow will, therefore, limit itself to a consideration of recent work.

NUCLEOPROTEINS

Generally applicable methods for the isolation of genuine nucleoproteins are not yet available. Not only does each cellular species have to be treated as a separate case, but since the stability conditions of these conjugated proteins are largely unknown, the relatively drastic treatment necessary for their detachment may often lead to artifacts, e.g., to the combination of the dissociated nucleic acid with the most basic protein component present in the extract. If one were to take an extreme view, one might state that only certain plant and bacterial viruses and some of the particulate lipid-pentosenucleoprotein complexes isolated by fractional centrifugation of cell extracts represent genuine nucleoproteins.

The composition of cell nuclei or of material prepared from them has been investigated in several laboratories. Euler *et al.* (2) described the isolation and fractionation of nuclei from calf thymus, pig liver, and from the Jensen sarcoma of the rat. Irradia-

² Many authors, unfortunately, still persist in considering desoxyribose and ribose as generic names. It goes without saying that designations, such as desoxyribonucleic acid, should be reserved for compounds whose sugar constituent has been identified as desoxyribose.

tion of thymus nuclei with x-rays failed to change the characteristics of the nucleoproteins isolated from them (3). Several studies on the composition and physical properties of thymus histone (4) and histone nucleate (5, 6) do not yet permit definite conclusions. Stern and co-workers (7) described briefly the isolation from thymus of a desoxyribonucleoprotein, carrying the unfortunate name genoprotein T, which they consider to be in the native state; this material is said to have a molecular weight of the order of a million. On the other hand, a molecular weight of 70,000 to 80,000 was assigned to a nucleoprotein preparation made by the extraction of thymus with *M* NaCl (6).

Mirsky & Pollister have continued their extensive investigations of desoxypentose nucleic acid complexes with protamines and proteins. Thus, a nucleoprotamine from trout sperm, comprising 91 per cent of the defatted nucleus, was described (8) as well as a group of nucleoproteins isolated from liver, kidney, thymus, spleen, pancreas, brain, testes, and pneumococci by extraction with *M* NaCl (9). The name "chromosin" proposed for these products, which contain desoxypentose nucleic acid, histone, and other proteins, should not be confused with the "chromosomin" of Stedman (10). The isolation of chromatin threads, considered as actual chromosomes, and some of the chemical components of this material have likewise been described (11, 12).

Whatever corrections the future might impose on some of the optimistic findings reported above, it certainly should be welcomed that at last chemists rushed in where cytologists feared to tread.

Other studies on nuclear proteins include an attempt to fractionate the proteins of the nucleus of chicken erythrocytes by ultracentrifugation (13), and considerations on the possible role of nucleoproteins as the structural proteins of the cell (14 to 16).

Investigations of the cytoplasmic lipid-pentosenucleic acid-protein complexes of very high particle weight have also been extended. The granules obtained from the cytoplasm of a variety of cells were studied by Brachet & Jeener (17), those from rat and guinea pig livers by Claude (18). The composition and properties of the thromboplastic protein fractions of beef lungs (19), human lungs, and placenta (20), which belong to this group of substances, and the distribution of thromboplastic activity in different cellular fractions (21) were likewise discussed.

The manner in which, in the nucleoproteins, the nucleic acids

are attached to the proteins still is completely obscure. In general, the pentosenucleoproteins of the cytoplasm appear to be somewhat more stable than the conjugated proteins of the nucleus. It is possible that in the latter the giant fibers of the polymerized desoxypentose nucleic acid are combined with proteins of a relatively low molecular weight, whereas in the cytoplasmic particles a lipoprotein of an enormous particle weight carries comparatively low-molecular pentose polynucleotides.

In this connection, mention should be made of several studies in which the interactions between proteins and nucleic acids were examined (22 to 24). Björnesjö & Teorell (23) studied the formation of complexes between thymus desoxyribonucleic acid and egg albumin or histone and attempted to define the conditions governing the relative proportions of the components in the precipitates. The results were discussed in the light of present conceptions of antigen-antibody combination.

Although nucleic acids of microbial origin have been studied repeatedly in the period under review, only one brief report on the isolation of a desoxypentose nucleoprotein from avian tubercle bacilli appears to have been published (25). A nucleic acid preparation isolated by Knight (26) from purified influenza virus with the use of strong alkali consisted of a mixture of pentose and desoxypentose nucleic acids. In purified preparations of the T_2 bacteriophage of *E. coli*, Taylor (27) found about 40 per cent of desoxypentose nucleic acid together with small amounts of pentose nucleic acid; Cohen & Anderson (28) reported only desoxypentose nucleic acid. The surface structure of the same bacteriophage was studied with the help of streptomycin which was found to form insoluble complexes with desoxypentose nucleic acids. These studies and investigations of the action of the pancreatic depolymerase suggested that about 30 per cent of the phage nucleic acid was localized on the surface of the particles (29). Precautions necessary for the interpretation of the ultraviolet absorption spectra of virus solutions were emphasized by Schramm & Dannenberg (30).

DESOXPENTOSE NUCLEIC ACIDS

Several important reports on the preparation and structure of the desoxypentose nucleic acid of calf thymus were recently published from Gulland's laboratory. Gulland, Jordan & Threlfall

(31) prepared this nucleic acid via the nucleoprotein which was extracted from the washed tissue with 10 per cent sodium chloride and split by means of chloroform. The sodium thymonucleate obtained had a purine nitrogen to pyrimidine nitrogen ratio of 1.6 and approached also in its elementary composition the analytical expectations derived from a study by Gulland, Jordan & Taylor (32) of the electrometric titration curves given by the same preparation. This work made it appear probable that the material contained, for every four atoms of phosphorus, 1.0 guanine, 1.0 thymine, 1.0 to 1.2 cytosine, and 1.0 to 0.8 adenine radicals. It must, however, be borne in mind that the degree to which individual nucleic acid preparations are homogenous cannot yet be stated and that, moreover, in view of the inadequacy of the available analytical methods, the presence in nucleic acids of small amounts of hitherto unidentified constituents cannot be excluded.

Ahlström *et al.* discussed the analytical composition and some of the physical properties of depolymerized desoxypentose nucleic acid preparations from cattle thymus and spleen (33) and of relatively undegraded fractions prepared from the nuclei of cattle thymus and liver and of pig liver (34). The mechanism of the hydrolysis of depolymerized thymus nucleic acid by 80 per cent acetic acid and the influence exerted by the split products on the rate of hydrolysis were investigated by Fonó (35).

An electrometric study of a highly purified sample of sodium thymonucleate led Gulland *et al.* (32) to the conclusion that in this polynucleotide the purine-pyrimidine hydroxyl groups and some of the amino groups are blocked, probably by hydrogen bonds between these groups. These authors postulated a chain structure for this acid with only very infrequent branching. Snellman & Widström (36) and Creeth *et al.* (37) studied the streaming birefringence of thymus nucleic acid. Creeth *et al.* also investigated the viscosity behavior of solutions of this substance and found additional evidence for the occurrence of hydrogen bonding. Other studies dealt with the decrease in viscosity of nucleic acid by irradiation with x-rays (38) or by the action of nitrogen mustards (39), which were also found to produce spectral changes (40), and with the influence of polymerized thymus nucleic acid on the sedimentation velocity of erythrocytes (41).

The desoxypentose nucleic acids which are active as transforming agents in bacteria will be discussed later. At this place, men-

tion may be made of the preparation from yeast cells of a highly polymerized desoxypentose nucleic acid (about 0.04 per cent of the wet cells) (42) and of the isolation of nucleic acid from *Rickettsia prowazeki* (43, 44).

PENTOSE NUCLEIC ACIDS

Progress in our knowledge of the chemical structure of yeast ribonucleic acid has not been considerable during the period under review. A growing conviction of the heterogeneous and badly degraded character of the yeast nucleic acid preparations available commercially is indicated by the numerous purification procedures found in the recent literature (45 to 51). Gulland & Walsh (52) studied the liberation of inorganic phosphate by the action of Russell viper venom on ribonucleic acid before and after degradation by alkali and again attempted to explain the existence of alkali-labile linkages in the polynucleotide by assuming the involvement of C_2 of the sugar. This view had to be relinquished, however, when Gulland & Smith (53) synthesized uridine-2-phosphate and showed it to have approximately the same stability toward hot alkali as uridylic acid. That D(-)-ribose is the carbohydrate constituent of the purine nucleotides derived from yeast pentose nucleic acid has again been confirmed (54).

Pentose nucleic acids also were prepared from other sources, viz., from sheep liver (55), from the larvae of *Calliphora erythrocephala* (56, 57), from barley root (58), and from *Spirillum volutans* (59). Guanylic, uridylic, and cytidylic acids, isolated from the nucleic acid of tobacco mosaic virus, were shown to be identical with the analogous nucleotides prepared from yeast ribonucleic acid (60).

NUCLEIC ACIDS AS CELLULAR CONSTITUENTS

Localization within the cell.—Davidson & Waymouth continued their studies on the pentose nucleic acids of liver (61, 62, 63). Their investigations (62), as well as those of Kosterlitz (64, 65, 66), point to the metabolic instability of the pentose nucleic acid-protein complexes of the liver cytoplasm. The changes, produced by fasting, in the distribution of nucleoproteins in the liver cell were also followed by ultraviolet microscopy (63, 67). The distribution of nucleic acids in the various fractions obtained by the differential centrifugation of lung extracts was likewise studied

(21). Harvey & Lavin (68) reported on the chromatin and the cytoplasm of *Arbacia* eggs, examined by means of ultraviolet photography.

Turnover and growth.—The turnover of the desoxypentose nucleic acids was in most cases investigated with the help of radioactive phosphorus. Andreasen & Ottesen (69) studied the rate of renewal of nucleic acid in the lymphoid organs of albino rats of different age groups and observed the greatest turnover in the thymus where as much as 5 to 6 per cent was newly synthesized in mature animals within three hours. For the lymph nodes and the spleen the corresponding figures were 1 to 2 per cent. In most other organs of the adult animal the rate of renewal of desoxypentose nucleic acid is very low, with the exception of the intestinal mucosa (70). The speed at which pentose nucleic acid, however, is newly formed is very considerable. Hammarsten & Hevesy (71) found, for instance, that rat liver renewed 3.3 per cent pentose nucleic acid within two hours, but only 0.1 per cent of desoxypentose nucleic acid. In regenerating rat liver Drabkin (71a) found a rapid new formation of pentose nucleic acid which was relatively independent of dietary protein.

Our understanding of the manner in which nucleic acids are utilized by, and incorporated into, the living cell still is in its beginning. That chromatin material labeled with radioactive phosphorus is rapidly taken up by the liver nuclei was shown by Marshak & Walker (72). Ahlström *et al.* (73) followed the metabolic fate of labeled depolymerized desoxypentose nucleic acid and found, two hours after the injection, a large part of the phosphorus tracer in the liver, mostly in the acid-soluble fraction.

Studies on the effect of the irradiation with x-rays appeared to provide evidence of a reduction in the turnover rate of desoxypentose nucleic acid (74 to 77). However, some of these results may have been due to the experimental arrangement rather than to a specific interference with the nucleic acid metabolism, as was recently pointed out by Ahlström *et al.* (78).

The effect of tissue extracts on the nucleoprotein phosphorus content of fibroblast cultures was examined by Davidson & Weymouth (79, 80). The very extensive investigations of Thorell (81, 82) on the mechanism of blood cell production utilized microspectrographic methods for the study of nucleic acid metabolism [see also (83)].

Cytochemical investigations include a study of changes in the localization of desoxypentose nucleic acid in the nucleus at the beginning of meiosis (84), a consideration of the synthesis of this compound in the course of embryonic development (85), and a report on the nucleolar inclusions in growing oöcyte cells (86). The nucleic acid metabolism in insects was followed by Rosedale (87).

Malignant growth.—It is quite clear that the chemical methods available at present for the characterization of nucleic acids are not refined enough to permit structural distinctions to be drawn between the substances present in normal and in cancer tissue. Most of the efforts have, therefore, been devoted to revealing merely quantitative differences. Studies by Davidson & Waymouth (88) on Rous sarcoma and on a chemically induced fowl tumor, and by Khouvine & Grégoire (89) on rat epithelioma point to a relatively high level of pentose nucleic acid in these tissues. The ratio of pentose to desoxypentose nucleic acid was four to six in the sarcoma and fowl tumor (much lower in human tumors) (88); in rat epithelioma the reverse relationship appears to exist (90). Analyses of a variety of normal and malignant tissues were also carried out by Schneider (91, 92). The nucleic acid metabolism of the Jensen sarcoma of the rat (78) and the effect on it of x-ray irradiation (78, 93) formed the subject of other studies. Stowell reported on the effect of x-rays on the desoxypentose nucleic acid content of transplantable carcinomata (94) and also published nucleic acid analyses of human tumors (95). The changes in the desoxypentose nucleic acid concentration of chromatin threads, isolated at different stages of carcinogenesis, were followed by Gopal-Ayengar & Cowdry (96).

Microorganisms and viruses.—Conditions affecting the nucleic acid metabolism in yeast were studied by Jeener & Brachet (97). The existence in bacteria of structures resembling cell nuclei has repeatedly been demonstrated in the recent past (98, 99, 100). The relationship between nucleotide metabolism and growth of the bacterial cell was investigated by Malmgren & Hedén (101). Vendrely and co-workers (48, 102, 103) reported on the desoxypentose and pentose nucleic acid content of a number of microorganisms, Petrik (104) on that of acid-fast bacteria. Desoxypentose nucleic acid was found to be released preferentially in the course of bacterial autolysis (105). Henry *et al.* (106, 107) continued their studies on the magnesium pentose nucleoproteins thought by them to be the carriers of the Gram stain.

Gratia *et al.* (108, 109), comparing the composition of the silk worm jaundice virus particles with that of granules obtained by the ultra-centrifugation of noninfected and infected tissue extracts, found the virus to contain only desoxypentose nucleic acid; in the granules isolated from healthy silk worms only pentose nucleic acid could be demonstrated, whereas the granules isolated from the infected worms contained a mixture of both nucleic acids.

NUCLEOTIDES

The structure of adenosine di- and triphosphate, originally formulated by Lohmann, in which the phosphoryl or pyrophosphoryl residue, respectively, is united with adenosine-5-phosphate through the stable 5-phosphoryl group, has been confirmed several times (110, 111, 112). The very elegant synthetic methods developed by Todd and his associates in the past few years led to the synthesis of muscle adenylic acid and adenosinediphosphate (113). A method for the differentiation of muscle and yeast adenylic acids by means of their acridine salts also was described (114). Gulland & Smith (53) reported the synthesis of uridine-2-phosphate. Other investigations included studies on the adenine nucleotides of liver (115, 116) and on the mechanism of decomposition of muscle adenylic acid during tissue autolysis (117).

NUCLEOSIDES

Todd and his associates continued their extensive series of studies on the synthesis of purine and pyrimidine nucleosides (118 to 124). The configuration at C₁ of the sugar in adenosine, uridine, and cytidine was determined by an indirect method. These nucleosides were shown to be the β -D-ribofuranosides (119, 122). Further evidence that in adenosine the ribofuranose residue is located at N₉ of the purine was provided (123). Howard *et al.* (124) synthesized cytidine by the condensation of acetobromo-ribofuranose with 2,6-diethoxypyrimidine, followed by the treatment of the reaction product with ammonia in methanol.

PURINES AND PYRIMIDINES

Synthetic methods for the preparation of adenine (125) and of thymine and 5-methylisocytosine (126) and procedures for the separation of purines in minute amounts (127) have been described. The ultraviolet absorption spectra of thymine (128) and of cytosine and isocytosine (129) were compared at different pH

values. Weil-Malherbe (130) studied the solubilization of polycyclic aromatic hydrocarbons brought about by the chemical interaction between a hydrocarbon molecule and one or two purine molecules. Peculiar effects with regard to the quenching of the fluorescence of polycyclic hydrocarbons and other fluorescing compounds by purines also were discovered (131).

The purine metabolism of slices of rat liver and kidney was studied by Bernheim & Bernheim (132). Oparin & Gelman (133) followed the formation of purines in germinating wheat seeds. Dietary adenine, in contrast to guanine, has been shown to be incorporated into the nucleic acids of the rat (134). Evidence that 5(4)-amino-4(5)-imidazolecarboxamide is a precursor of purines in *E. coli* (135) and oxaloacetic acid a precursor of pyrimidines in *Neurospora* (136) has been presented.

BIOLOGICAL EFFECTS

Microbial transformation.—The isolation, by Avery *et al.* (137), of a desoxypentose nucleic acid fraction from pneumococcus Type III, capable of inducing transformation of pneumococcal types, was followed by several publications from the same laboratory. The original method for the preparation of the transforming agent was improved and extended to pneumococcus Types II and VI (138). Certain properties of these biologically active substances appear to bear out their recognition as highly polymerized specific desoxypentose nucleic acids, others would seem to speak against it. A purified preparation of desoxyribonuclease from beef pancreas (139) has been shown to be able, in very minute amounts, to bring about the inactivation of the transforming substance (140). But the enzyme preparation used was not entirely free of proteolytic enzymes and may have contained other, as yet unrecognized, enzymes. Other findings cannot be entirely reconciled with the present views on nucleic acid structure, e.g., the inactivation of the transforming agent by drying in the frozen state (137), and its reversible inactivation by ascorbic acid in the presence of copper (141). Another baffling fact is the requirement for pneumococcal transformation *in vitro* of several factors shown to be present in serum (142).

A similar phenomenon in the group of colon bacilli was later discovered by Boivin and co-workers (143). Here, too, the transforming substance appeared to be a nucleic acid of the desoxy-

pentose type, although not as highly purified as the pneumococcal agents (144). Later publications described some of the conditions necessary for the transformation (145) and attempted to draw theoretical conclusions (146).

Other biological effects of nucleic acids.—Many effects ascribed in the literature to nucleic acids will be found on closer examination to be due to their decomposition products. It is truly regrettable that several authors appear to be unwilling to distinguish between nucleic acids, nucleotides, nucleosides, and the constituents of the latter.

Pentose nucleic acids or the pentose nucleic acid-lipoprotein granules of the cytoplasm appear to play a role in the evocation of the neural tube in young amphibian gastrulae; but the mechanism and the specificity of this effect are far from clear [compare Brachet's review (147)].

Chalkley & Greenstein (148) described the effects of desoxy-pentose nucleic acid and pentose nucleic acid on the rate at which methylene blue is decolorized by various tissue extracts. The interpretation of the numerous observations made is not easy [compare also (149)]. Thymus nucleic acid, yeast ribonucleic acid, and ribonucleotides were found by Zittle (150) to inhibit succinic dehydrogenase. Carter & Greenstein (151) reported on a protective effect of thymus nucleic acid on the heat coagulation of egg albumin.

Nucleotides.—A consideration of the role of adenylic acid, adenosinediphosphate, and triphosphate in enzymatic reactions is, of course, outside the scope of this review. Tissue changes in mice produced by treatment with pentose nucleotides were described briefly by Barker *et al.* (152) and in greater detail by Parsons *et al.* (147a). In tumor-bearing animals, adenylic and guanylic acids caused inhibition of tumor growth; uridylic acid promoted growth, cytidylic acid had little effect. Adenylic and cytidylic acids decreased the size and weight of the spleen; guanylic and uridylic acids had the opposite effect. The systemic changes in normal mice brought about by the pentose nucleotides were similar to those produced by carcinogenic compounds, by the growth of sarcomata, or by x-ray irradiation. Hutchings *et al.* (153) found yeast nucleic acid and the mononucleotides derived from it highly active as growth factors for *Lactobacillus gayonii*. While cytidylic acid was the most active nucleotide, it is noteworthy that a yeast nucleic

acid hydrolyzate proved more active than the purified nucleotides; guanosine was inactive.

Purines and pyrimidines.—The very interesting reports of Colowick & Price on the function of guanine as a coenzyme in enzymatic transphosphorylation (154) and on the enzymatic liberation of guanine from ribonucleic acid by a "ribonucleic acid phosphorylase" present in rat muscle (155) appear to have been retracted by one of the authors (156). Frommeyer *et al.* (157) found thymine, given orally in massive doses, to be a potent antianemic substance in patients with pernicious anemia in relapse. Field *et al.* (158) observed that the oral administration of adenine or xanthine raised the plasma fibrinogen level in rabbits. While Lecoq *et al.* (159) consider adenine as one of the indispensable factors of the vitamin B complex necessary for the correction of the neuromuscular disorders produced by total B complex avitaminosis, Raska (160) found that the addition of adenine (400 to 500 mg. daily) to the diet of dogs resulted in the production of a multiple avitaminosis showing all the symptoms of experimental pellagra. The same author also studied the effect of adenine on tumor growth (161). Leone (162) studied the effect on rats of a diet high in purines and found an increased elimination of allantoin and uric acid. Wilson (163) studied the tolerance of *Drosophila* larvae for thymine and obtained evidence that this pyrimidine was involved in the pigment metabolism.

Numerous reports have appeared on purines and pyrimidines as essential factors in microbial nutrition, although this field continues to be plagued by a considerable amount of vagueness. The purine and pyrimidine requirements of the protozoan *Tetrahymena geleii* were described (164). Fries (165) reported on the production of x-ray induced mutants of the fungus *Ophiostoma multianellatum* requiring adenine, guanine, hypoxanthine, and uracil respectively. The essential nature of purines and pyrimidines for certain mutants of *Neurospora* was discussed by Pierce & Loring (166). Adenine-requiring mutants of *Neurospora* were compared in their biochemical and genetic characteristics by Mitchell & Houlahan (167). Ryan *et al.* (168) described a mutant of *Clostridium septicum* which required uracil. Lampen *et al.* (169) studied an x-ray induced mutant of *E. coli* which required *p*-aminobenzoic acid for growth. This growth factor could, however, be replaced by a mixture of thymine, adenine, xanthine, and amino acids. The production by

nitrogen and sulfur mustards of *E. coli* mutants requiring purines or pyrimidines was reported by Tatum (170).

The ability of nucleic acids and of their constituents to support growth of Group A streptococci was studied by Wilson (171) who found yeast nucleic acid and depolymerized thymus nucleic acid, xanthine, guanine, hypoxanthine, and the adenine and guanine nucleosides active, whereas highly polymerized thymus nucleic acid, adenine, uracil, cytosine, and thymine were ineffective. Roblin *et al.* (172) prepared the triazolopyrimidine analogues of adenine, hypoxanthine, guanine, and xanthine and tested their growth-inhibiting effect on *E. coli* and *Staph. aureus*. The antagonistic action was reversed by the analogous purines. The effect of thymine on *L. casei* (173, 174) and on *S. faecalis* (174) also was studied. The inhibiting effect of thiouracil and thiothymine on *L. casei* and *E. coli* was reversed by the corresponding pyrimidines; however, thiothymine was ineffective in media containing folic acid (175). Other studies dealt with a growth-stimulating effect of guanine on *Staph. aureus* (176), with the influences on *E. coli* growth of adenine and uracil (177), and with an antisulfonamide effect of adenosine (178). Schopfer (179) found the bacteriostatic action of sulfathiazole to be opposed by nucleic acids and by adenine (the pyrimidines were inactive) and discussed the sulfonamide effect as an interference with the biosynthesis of purines.

ENZYMES

Depolymerases and other enzymes attacking desoxypentose nucleic acids.—To describe an enzyme solely in terms of a physical change which it produces in the substrate is precarious, since a large number of chemical reactions may be hidden behind as simple sounding a description as depolymerization; but in the case of the nuclear desoxypentose nucleic acids the growth and the cleavage of the giant fibers conceivably is of great biological importance, and enzymes which reduce the high degree of asymmetry of these substances may provisionally be classified under the term depolymerase.

An enzyme, termed desoxyribonuclease, very active in decreasing the viscosity of thymus desoxyribonucleic acid and of other desoxypentose nucleic acids, was isolated in a purified form from beef pancreas by McCarty (139). This enzyme required magnesium (or manganese) and was, therefore, inactivated by such anions as

citrate or fluoride. It also was found to be inhibited by the specific antibody elicited by the immunization of rabbits with the enzyme. Carter & Greenstein (180) found that the viscosity drop of thymus nucleic acid, produced by this enzyme, was accompanied, in the presence of a number of activators (magnesium, manganese, calcium, arginine, etc.), by the liberation of dialyzable fragments. At the same time, roughly one acid group was liberated per four phosphorus atoms (180 to 182). Laskowski *et al.* discussed the isolation (183) and the assay (184) of the pancreas depolymerase and showed that a crystalline protein prepared from beef pancreas (185) owed its depolymerizing activity to an impurity (186).

Whether there exists a necessary sequence of enzymatic degradation, i.e., whether the highly polymerized desoxypentose nucleic acid must first be depolymerized to smaller polynucleotide fragments before forming the substrate for further dephosphorylation, deamination, etc., does not yet become clear from an inspection of the literature which on this point, as on so many others, is both verbose and fragmentary. Experiments with crude tissue extracts (187, 188) may give a picture of the over-all fate of these complicated substances, but will hardly lend themselves to an elucidation of their structure. According to Zittle (182) thymus nucleic acid is slowly hydrolyzed by a phosphoesterase from calf intestinal mucosa, but this action is more rapid and complete after the preliminary treatment of the nucleic acid with the pancreas desoxyribonuclease. The latter enzyme failed, however, to act on a nucleic acid prepared with the aid of strong alkali (189). The experiments on the hydrolysis of both types of nucleic acids by the intestinal phosphoesterase were summarized by Zittle (190).

In this connection, a study by Cohen (191) of the degradation of thymus nucleohistone by proteolytic enzymes may be mentioned in which changes in amino acid composition and viscosity were correlated with the progress of proteolysis.

Ribonucleases.—Kausche & Hahn (192) briefly described a method for the crystallization of ribonuclease from beef pancreas. Kunitz (193) presented a spectrophotometric method for the measurement of ribonuclease activity based on the production by this enzyme of a gradual shift towards the shorter wave lengths in the ultraviolet absorption spectrum of yeast ribonucleic acid. Lea *et al.* (194) investigated the inactivation of ribonuclease by irradiation with x-rays and directed attention to the relationship between the

particle size of the enzyme and the target size [compare also p. 177 of Lea's recent book (195)].

Attempts have been frequently made to follow the mechanisms of the enzymatic degradation of ribonucleic acid and to utilize the findings for the elucidation of nucleic acid structure. While experiments of this nature continue to be handicapped by the lack of substrate uniformity, the introduction of purified enzymes in the recent past represents great progress. It has been known since the fundamental work of Kunitz (196) on the crystalline ribonuclease of beef pancreas that the bulk of the enzyme-resistant reaction product (197) consists of material of a relatively high molecular weight. Recent studies by Loring *et al.* (51) and Schmidt *et al.* (198) have contributed to a clearer understanding of the type of enzyme action that takes place. It would seem that the action of ribonuclease is exerted preferentially on the pyrimidine nucleotide portion of the nucleic acid.

Chantrenne who described a method for the preparation and fractionation of yeast ribonucleic acid (49, 50) studied the mechanism of the action of ribonuclease (199). His results indicate the presence (per tetranucleotide) of two secondary phosphoric acid groups, one of which presumably forms the connecting links in a larger molecule. The volume changes accompanying this enzymatic reaction were studied with a somewhat different substrate by Chantrenne *et al.* (200). The results, arrived at by microdilatometry, were interpreted as showing crystalline ribonuclease to act in two ways: (a) as a depolymerase (accompanied by an increase in volume); and then (b) as a phosphatase (with decrease in volume). Schramm *et al.* (201) also investigated the mechanism of ribonuclease action.

Other papers include studies on inhibitors of ribonuclease (202, 203), on the distribution of similar enzymes in blood and tissues (47, 204, 205, 206), and on the occurrence of ribonuclease in *Pasteurella pestis* (207). Stowell (208) discussed the use of ribonuclease in the histochemical study of fixed tissues. Schlamowitz & Garner (209) described the preparation of a ribonuclease from soybeans.

Nucleotidases.—Histochemical studies on the localization of enzymes able to dephosphorylate nucleotides were reported by Dempsey & Deane (210). Enzyme systems occurring in a variety of animal tissues, and apparently similar to the enzymes discovered by Dische (211) in hemolyzed erythrocytes from human blood,

formed the subject of a study by Schlenk & Waldvogel (212). The disappearance of ribose from such compounds as guanylic, inosinic, adenylic acids, guanosine, and adenosine was shown to be due to an enzymatic process for which phosphoric acid was essential.

Nucleosidases.—A very interesting type of enzymatic reaction was described recently by Kalckar (213, 214). This phosphorolytic enzyme, designated nucleoside phosphorylase and prepared from liver (215), catalyzes the reaction: ribose-1-purine + phosphate \rightleftharpoons ribose-1-phosphate + purine. As purine nucleosides inosine or guanosine was employed. The acid-labile phosphopentose formed (isolated as the barium salt), when incubated with hypoxanthine or guanine together with the nucleoside phosphorylase, gave rise to purine nucleoside.

Deaminases.—In connection with the development of a series of micromethods for the analytical determination of purines, to be mentioned in the next section, Kalckar (215) described procedures for the preparation of adenylic acid deaminase, adenosine deaminase, and guanase. Mitchell & McElroy (216) reported on the preparation of an adenosine deaminase from *Aspergillus oryzae* and discussed some of its properties. Schaedel *et al.* (217) found isoguanosine (2-hydroxy-6-aminopurine-D-riboside) and adenine thiomethylpentoside not to be deaminated by adenosine deaminase from small intestine, nor were these substrates attacked by nucleosidases. A study of the specificity of guanase from rabbit liver showed that of a number of substituted guanines and other purines and pterins only 1-methylguanine was attacked (218).

ANALYSIS

The lively interest in nucleic acids and in their role in cellular proliferation and growth phenomena has, of course, prompted the elaboration of a number of analytical procedures for the quantitative estimation of nucleic acids in tissues and in isolated preparations. These methods are, in general, based on one or on a combination of several of the following principles: (a) isolation and fractionation of polynucleotides; (b) ultraviolet spectroscopy; (c) colorimetric sugar estimation; (d) phosphorus determination; (e) direct or indirect determination of purine and pyrimidine constituents; (f) histochemical procedures. Almost all methods available so far lack in precision and ease of execution and several are to some extent affected by the interference of other compounds present, such as amino acids, carbohydrates, etc.

Hammarsten (219) recently made a detailed study of the extraction and separation of cellular polynucleotides which represents an interesting combination of several of the principles mentioned above. The thoroughly disintegrated cell material was extracted with strong salt solutions containing urea and the polynucleotides were precipitated as copper salts. The separation of the pentose and desoxypentose nucleotides made use of the solubility of the sodium salts of the latter in aqueous phenol or of differences in stability towards alkali. Analyses of yeasts and of rat liver, intestine, and spleen are included.

Schneider (220, 221) extracted the nucleic acids from tissue material with hot trichloroacetic acid and applied conventional methods for the analysis of the extract. The use of spectrophotometry in the ultraviolet for the determination of the nucleic acid content of nucleoproteins was discussed by Euler & Högberg (222).

While the conviction appears to be gaining ground that the absence of a color reaction for desoxy sugar does not necessarily signify the presence of ribose nucleic acid in ill-defined natural products, there is not yet agreement on what a positive test denotes. That most colorimetric sugar reactions are impeded by the presence of proteins is, of course, well known [compare, for instance, for the diphenylamine reaction (33, 223)]; but often less attention is paid to interference by other sugars and to the divergent stabilities of different nucleotides. The color reaction of desoxypentose with cysteine and sulfuric acid, discovered by Dische (224), has recently been proposed as a quantitative procedure applicable in the presence of ribonucleic acid (225). Similarly, the phloroglucinol reaction of pentoses has been suggested (226) as a method for the quantitative estimation of pentose nucleic acid, preferable to the use of orcinol, and has been applied to the study of yeast cells (227).

A color reaction of desoxypentoses with tryptophane in the presence of perchloric acid is said to permit, under certain conditions, the estimation of both purine-bound and pyrimidine-bound sugar (228) [compare also the discussion of this test in (220)]. Other publications discuss the use of the orcinol reaction for the estimation of purine nucleosides (229) and of pyrimidine nucleosides (230) as well as for distinguishing between ribose-3-phosphate and ribose-5-phosphate (231).

A different principle was applied by Schmidt & Thannhauser (232) to the quantitative determination of desoxypentose and

pentose nucleic acids and of phosphoproteins in animal tissues. Their method is based on the differences in stability towards weak alkali exhibited by these compounds. Jeener & Brachet (233) described a procedure for the estimation of nucleic acids in yeast cells which made use of the combination of these substances with a basic dye (toluidine blue).

Methods for the separation of purine nucleosides from free purines were discussed by Kerr & Seraidarian (234). Loring *et al.* (235) analyzed several preparations of yeast ribonucleic acid. Some of the preliminary results reported were quite surprising. The crude yeast nucleic acid and also a nucleic acid preparation from tobacco mosaic virus contained twice as much adenine as guanine and two to three times as much purines as pyrimidines.² The analyses were carried out by means of *Neurospora* mutants requiring adenine or pyrimidine nucleosides or nucleotides. Cytidine and cytidylic acid were separated from uridine and uridylic acid, respectively, by means of the insoluble phosphotungstates of the first-mentioned compounds.

With respect to the analysis of mixtures of free purines, a new approach to the problem has been made possible by the elegant work of Kalckar (237, 238). His method makes use of differential spectrophotometry by following the changes in the ultraviolet absorption of purines while acted upon by specific enzymes. In this manner, hypoxanthine, inosine, xanthine, guanine, guanosine, uric acid (237), and adenosine, adenosine-5-phosphate (also together with adenosine-3-phosphate), and adenosinediphosphate and triphosphate (238) were estimated in very small quantities. Kalckar (215) also described the preparation of the enzymes required by his procedures.

A different approach, namely partition chromatography, was chosen by the present writers for the separation of minute amounts of purines (127) and pyrimidines and for their quantitative spectrophotometric estimation following separation (unpublished results). By this procedure, adenine, hypoxanthine, guanine, xanthine, uracil, cytosine, and thymine could be separated.

Conditions governing the estimation of purine nitrogen were

² Some of these results seem to require correction since, as appears from a recent note (236), the adenine-requiring mutant of *Neurospora*, used in the assays, misbehaved.

discussed by Vendrely and co-workers (239, 240). An interesting preliminary paper by Stacey *et al.* (241) attempted to clarify the chemical reactions that form the basis of two important color reactions, viz., Feulgen's fuchsin and Dische's diphenylamine tests. It would appear that ω -hydroxylevulinic aldehyde, formed from the desoxy sugar, is the intermediate responsible for the color reactions. The specificity of the Feulgen reaction as a histochemical tool has been discussed by several authors (242 to 245).

REVIEWS

The papers read at a symposium on nucleic acids, held at Cambridge in 1946, have been published as a book (see 147, 147a). Another symposium volume on nucleic acids and nucleoproteins appeared too late for consideration in this article (246). A very interesting monograph by Lea (195) contains much information pertinent to the subject discussed here.

A detailed survey of our knowledge of nucleoproteins was presented by Greenstein (247); nucleoproteins and genes formed the subject of two briefer reviews (248, 249). Stedman (10) discussed the chemistry of cell nuclei, Davidson (250, 251) the biochemistry of nucleic acids and nucleoproteins. The chemistry of nucleic acids was reviewed by Tipson (252), their metabolism by Hevesy (253), their function as pneumococcal transforming agents by McCarty (254). Desoxypentose nucleic acids in tumors were discussed by Stowell (255). A survey of the problem of the synthesis of nucleotides was presented by Todd (256).

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CARBOHYDRATE METABOLISM¹

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This review is designed to cover carbohydrate metabolism in animals and includes only such aspects of comparative biochemistry as are pertinent to the discussion of this topic.

HIGH ENERGY PHOSPHATE BONDS

Evidence continues to accumulate that the reactions of glycolysis and of the tricarboxylic acid cycle probably give a picture, correct at least in outline, of the pathway over which the bulk of the carbohydrate of the animal is metabolized. When ingested glucose is oxidized in the body over this route, it leaves behind, besides carbon dioxide and water, an accumulation of chemical energy stored in the pyrophosphate bonds of adenosinetriphosphate (ATP), which may then serve as a source of chemical energy to drive other reactions.

Lipmann (1) and Kalckar (2) have discussed the function of phosphate in syntheses, and Umbreit (3) has reviewed the general occurrence of energy transfer by way of phosphate in all living forms. Albaum & Ogur (4) have added a new adeninepentosepyrophosphate from plants to the list of biologically active nucleotides. Reports have appeared (5, 6, 7) on the interesting accumulation of metaphosphate in biological systems.

The manner in which the energy of the pyrophosphate bonds of ATP can be transformed into the energy of muscular contraction continues to be a focus of interest. Engelhardt has reviewed the contributions of the Russian school (8), and more recently Szent-Györgyi (9) has summarized the extensive work done on this subject at his laboratory during the war years. The discovery of the muscle protein, actin, by Straub, the crystallization of myosin, and the elucidation of the interaction of actin and myosin constitute some of the more significant advances reported by the Hungarian group. Jakus & Hall (10) and Astbury and co-workers (11) have made electron microscope studies of the various particles involved. The discussion by Astbury (12) of the structure of fibres in muscle

¹ The period covered in this review extends from October, 1946 to November 1947.

is also pertinent. The chemical detail of the action of ATP with these muscle proteins does not yet seem clear, though experimental data bearing on this point have been contributed by a number of workers. Polis & Meyerhof (13) have reported a fractionation of myosin with a threefold increase in adenosinetriphosphatase activity. Bailey & Perry (14) have shown that the sulfhydryl groups of myosin are necessary for actomyosin formation and suggested that the degradation of actomyosin by ATP is due to the competition of ATP with actin for the myosin sulfhydryl groups. Buchthal *et al.* (15) have studied the volume contraction caused by application of ATP to myosin threads. Bate-Smith & Bendall (16) have confirmed the findings of Erdös [see (9)] in regard to the association of the stiffening of mammalian muscle during the onset of rigor mortis with a decrease in ATP. Fischer *et al.* (17) have noted a decrease in the adenosinetriphosphatase activity of denervated muscle.

General acceptance of the significance of high energy bond phosphate is reflected in the increased tendency to analyze tissues for phosphate compounds in attempts to correlate pathological or special physiological conditions with changes in phosphate bond generation or transfer (18 to 21). Of interest is an improved method devised by Potter (22) for measuring the capacity of a tissue to cause oxidative phosphorylation; also, a detailed study by LePage (23) of the effects of hemorrhage on phosphorylated metabolic intermediates of the tissues. In addition, Horvath & Tebbe (24) have reported that the muscles of scorbutic guinea pigs contain subnormal quantities of ATP, phosphocreatine and creatine and have suggested that this may account for the fatigability of such animals.

THE PHOSPHORYLATION OF GLUCOSE

The first step in the utilization of glucose is a phosphorylation mediated by the enzyme hexokinase. This is an irreversible reaction consisting of a transfer of phosphate from ATP to glucose to form glucose-6-phosphate and adenosinediphosphate (ADP). The Coris and their collaborators have demonstrated that this reaction may be controlled by insulin, pituitary and adrenal factors, and their results give promise of revealing, in part, the nature of the hormonal control of carbohydrate metabolism. Earlier results were reviewed last year (25). Colowick (26) has also recently summa-

rized some of the properties of the hexokinase system. Colowick, Cori & Slein (27) have published detailed results of their studies, the main points of which may be summarized as follows:

(a) Hexokinase freshly prepared from muscles of diabetic rats is not fully active. This inhibition of enzymatic activity is temporary. It can be increased by an adrenal cortex preparation and completely relieved by insulin.

(b) Hexokinase prepared from muscles of normal rats is not affected by insulin, but addition of an anterior pituitary extract causes an inhibition which can be intensified by adrenal cortex extracts and completely relieved by insulin. The active adrenal and pituitary factors have not been identified. The preparation of the unstable, active pituitary fraction has been described. The authors feel that the transient inhibition of the hexokinase from diabetic rats is associated with the presence in the preparations of the pituitary factor. A major difficulty in the experimental investigation of these highly interesting phenomena lies in the instability of this preparation.

Broh-Kahn & Mirsky (28) have confirmed the inhibition of hexokinase activity by anterior pituitary extracts, and the relief of the inhibition by insulin but have reported failure to find inhibition of hexokinase in tissues of rats made diabetic with alloxan. They did not test the effect of adrenal cortex, however, and in view of the extreme lability of the inhibition, as reported by Colowick *et al.* (27), a certain proportion of negative results may be regarded as inevitable.

The action of insulin on the hexokinase reaction consists of a relief of an inhibition caused by a pituitary factor and enhanced by an adrenal factor. Insulin does not activate hexokinase from normal tissues. In contrast to this is the stimulation by insulin of the formation of glycogen from glucose by isolated diaphragm muscle from normal rats [originally described and studied by Gemmill and his collaborators (29)]. The effects of insulin on this system have been repeatedly confirmed. Stadie & Zapp (30) have examined the phenomenon systematically and determined the effects of pH, glucose concentration, ionic composition of the medium, and insulin concentration, among other variables. Krahll & Cori (31) have made a detailed study of the rate of glucose removal from the medium by diaphragms of (a) normal; (b) diabetic; (c) adrenalectomized; and (d) diabetic and adrenalectomized rats,

in each case determining the extent of stimulation of the glucose uptake by insulin. They found that diaphragms from diabetic rats showed an impaired ability to remove glucose and that insulin, though it stimulated the uptake, did not bring it back to normal. Adrenalectomy of diabetic rats restored the behavior of the diaphragms to normal. Diaphragms from adrenalectomized rats (not diabetic) also behaved like normal.

Using a similar system, Corkill & Nelson (32) found that injection of anterior pituitary extracts into rats, prior to removal of the diaphragms, abolished the stimulation of glucose uptake and glycogen deposition by insulin. Krahle & Cori feel that this may explain the inability of insulin to restore the activity of diabetic diaphragms to normal. Insulin apparently cannot effectively cancel the pituitary inhibition in the intact muscle *in vitro*, whereas it is able to do so in the hexokinase system where the cell structure is destroyed.

Verzár & Wenner (33), using Gemmill's system, have also reported that diaphragms from adrenalectomized rats behave like normal. In addition, they found that the stimulation of glycogen deposition by insulin was completely abolished by desoxycorticosterone. This could be attributed to the increased glycogen breakdown caused by the adrenal hormone, rather than to a direct inhibition of insulin action. However, Riesser (34) reported that desoxycorticosterone had no effect in the same system but that adrenal extracts and epinephrine completely abolished the stimulation by insulin. In addition, he noted no effect from acetylcholine, pituitary extracts, acetoacetic acid, vitamins B₁, B₂, and C, a slight stimulation by thyroxine, and inhibition by sodium fluoride and zinc acetate. Further developments in this field may be awaited with interest. It is not certain whether the effect of insulin in stimulating oxygen consumption of frog muscle, as reported by Fisher, Hall & Stern (35), may be a related phenomenon.

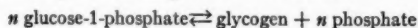
Racker (36) has devised spectrophotometric methods for measuring both hexokinase and phosphofructokinase. Colowick (26) has also discussed recent work on the latter enzyme, which catalyzes the transfer of phosphate from ATP to fructose-6-phosphate to form hexosediphosphate and ADP. The occurrence of hexosediphosphate in living yeast and its accumulation during normal fermentation has been reaffirmed by Suomalainen & Arhimo (37).

Additional work on the specificity of the enzymic phosphorylation of sugars by yeast has been done by Gottschalk, who has given

data to show that both α - and β -mannopyranose are fermented without preliminary interconversion by mutarotation (38), that the selective fermentation of D-fructose from invert sugar by Sauternes yeast is due to the greater permeability of the cell for fructose (39), that D-glucopyranose is fermented more rapidly than D-fructofuranose by yeast because the higher concentration of the glucopyranose outweighs the greater affinity of fructofuranose for the common enzyme (40). Gottschalk has also discussed the relationship of carbohydrate structure to enzyme specificity (41).

FORMATION AND BREAKDOWN OF THE GLUCOSIDE BOND

The precursor of glycogen in the animal body is glucose-1-phosphate, formed enzymically from glucose-6-phosphate. The synthesis of glycogen is catalyzed by the enzyme phosphorylase (plus a branching factor) according to the reaction:



as demonstrated by the Coris. Evidence is accumulating that many syntheses of glycoside linkages in nature occur in an analogous fashion, though there are marked differences in detail.

Notable advances in the study of glucoside bond formation, especially by the bacterial enzyme, sucrose phosphorylase, have been made by Doudoroff and his collaborators. Sucrose phosphorylase catalyzes the reversible reaction:



Hassid, Doudoroff & Barker (42, 43) and Meagher & Hassid (44) have studied the substrate specificity of muscle and potato phosphorylase as well as of bacterial sucrose phosphorylase. Maltose-1-phosphate, galactose-1-phosphate, mannose-1-phosphate, and xylose-1-phosphate are not acted upon by any of these enzymes. Sucrose phosphorylase can, however, act on other substrates than D-fructose. L-sorbose, L-arabinose, D-ketoxylase and L-ketoarabinose will all react with glucose-1-phosphate in the presence of the enzyme. The products of these reactions include several new disaccharides, one of which is reducing. Thus it can be seen that sucrose phosphorylase can transfer the glucoside residue of glucose-1-phosphate to a number of acceptors. Furthermore, Doudoroff, Barker & Hassid (45) found that a rapid exchange of radioactive phosphorus occurred between glucose-1-phosphate and inorganic phosphate incubated with the en-

zyme in the absence of any other sugar. In the absence of free phosphate the enzyme could also cause the synthesis of sucrose from glucosidoketoxylolide and fructose. On the basis of these results, the authors conclude that sucrose phosphorylase functions as a "transglucosidase" and acts by combining reversibly with glucose furnished as glucoside by a number of suitable donors, among which are glucose-1-phosphate as well as the sugars mentioned previously. The concept that an enzyme can act by first combining with a particular group of atoms furnished by a suitable donor and then transferring this group to a suitable acceptor (both reactions being reversible) is analogous to the concept of oxidation-reduction of the enzyme-prosthetic group in hydrogen transport. The clear-cut experimental evidence for this mechanism in the case of glucose transfer is gratifying. Doudoroff, Barker & Hassid (46) have also demonstrated an arsenolytic decomposition of sucrose and of glucose-1-phosphate, by sucrose phosphorylase, which they explain by the formation of an unstable glucose-1-arsenate. The action of arsenate in this instance is thus regarded as being analogous to the action of arsenate with glyceraldehyde phosphate dehydrogenase.

Further contributions to our knowledge of polysaccharide synthesis have been made by Hehre & Hamilton (47) who have demonstrated that certain bacteria of the *Neisseria* genus can convert sucrose to a glycogen or amylopectin-like polysaccharide with a liberation of fructose. Glucose-1-phosphate was barely active in the system. The authors point out the similarity of the system to those synthesizing dextrans and levans and suggest the name amylo-sucrase for the enzyme.

Another type of glycoside bond formation has been studied by Kalckar (48) who has reported on the properties of nucleoside phosphorylase from rat liver. The enzyme catalyzes the reversible conversion of inosine and guanosine to the corresponding free purine plus a pentose-1-phosphate, presumably ribose-1-phosphate.

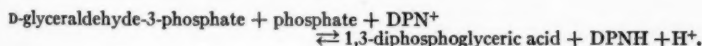
A number of studies of enzymes catalyzing the irreversible, hydrolytic cleavage of glucoside bonds have also appeared (49 to 55).

GLYCOLYSIS

In animal tissues the anaerobic conversion of carbohydrate to lactic acid by the well-known steps of glycolysis is now thought to be identical with the preliminary steps preceding the aerobic oxidation of glucose, except that pyruvic acid is considered to be the end point of the aerobic sequence of reactions.

Reiner (56) has studied liver and brain homogenates to determine the additions necessary to obtain maximal aerobic activity with glucose as a substrate. A careful analysis has been made by Meyerhof and his collaborators of the factors affecting the rates of anaerobic glycolysis. Meyerhof & Geliazkova (57) demonstrated that the concentrations of the enzymes hexokinase and adenylpyrophosphatase are the main factors determining turnover rate of sugar. The enzyme converting galactose into one of the fermentable hexoses was shown to be associated with the insoluble fraction of the tissue. Meyerhof (58) also demonstrated that the different rates of fermentation of glucose and fructose are related to the different affinity of hexokinase for these two sugars, and that the relative affinity varies with ATP concentration. In addition, Meyerhof & Wilson (59) have clarified previous confusion in regard to the different behavior of brain homogenates and brain extracts with respect to fermentation rates of hexosediphosphate and of free sugars. The adenylpyrophosphatase of homogenates is mainly associated with insoluble particles and is largely centrifuged out during the preparation of extracts. As a result hexosediphosphate is fermented more rapidly in homogenates than in extracts, while for the free sugars the opposite is true. A proper balancing of phosphate donors and acceptors is necessary in each case, in order to demonstrate the true capacity of the fermenting enzymes present. These studies serve to emphasize the precautions which must be taken in analyzing the significance of experimental observations on the rates of complex reactions. Previous reports of inactivity of hexosediphosphate as a glycolysis substrate in brain extracts were due, in Meyerhof's opinion, to ignorance of these factors.

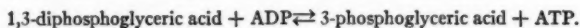
The oxidative reaction in fermentation has been studied in detail by Meyerhof & Oesper (60). They have demonstrated that the reaction obeys the law of a thermodynamic equilibrium in regard to all participants, and should be formulated:



When the equilibrium of this oxidation reduction was coupled with the equilibria catalyzed by isomerase and aldolase, it was again found, as shown previously, that the established concentration of glyceraldehyde phosphate was about 25 per cent higher than the equilibrium catalyzed by isomerase required. This is regarded as evidence for the existence of an unstable addition prod-

uct which is formed only in the presence of the oxidizing enzyme and cozymase.

The step in glycolysis which follows the oxidation is the transfer of phosphate to ADP according to the reaction:



Bücher (61) has purified and crystallized the enzyme from yeast which catalyzes this reaction. Magnesium ions are required. The equilibrium constant of the enzyme and the dissociation constant with respect to magnesium, ATP, diphosphoglyceric acid, and phosphoglyceric acid were accurately determined.

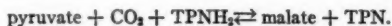
Respiration and glycolysis in gastric mucosa has been studied by Lutwak-Mann (62). Bird (63) has examined the relative contributions of erythrocytes and leucocytes to glycolysis in human blood, and Warren & Ebaugh (64) have determined the effect of various ions on glycolysis of rat liver. Respiration and glycolysis of isolated lung, and the effect of phosgene on these reactions has been studied by Simon, Potts & Gerard (65). The inhibition of glycolysis by adrenochrome in brain homogenates (66), in frog muscle (67), and in liver brei (68) has also been examined. Gemmill (69) has found that alloxan inhibits glycolysis in frog muscle extracts, and that the inhibition is reversed by cysteine. Chen & Geiling (70) have presented evidence that glycolysis in *Trypanosoma equiperdum* involves a phosphorylative process, thus disposing of another system previously thought not to involve such a mechanism.

THE FIXATION OF CARBON DIOXIDE

Both the addition of carbon dioxide to pyruvate to form a dicarboxylic acid, and the analogous addition of carbon dioxide to α -ketoglutarate to form a tricarboxylic acid, have been shown to occur as enzyme-catalyzed reactions in animal tissues. Ochoa's spectrophotometric evidence for the latter reaction has recently been confirmed by Grisolia & Vennesland (71) who employed radioactive carbon to demonstrate an enzymic exchange between carbon dioxide and the tertiary carboxyl group of isocitric acid. The two β -keto acid syntheses are the only carbon dioxide-fixing reactions at present known to be involved in the carbohydrate metabolism of the animal organism.

It has been thought previously that the primary product of the fixation was in each case a β -keto acid; oxaloacetate being formed from pyruvate and carbon dioxide, and oxalosuccinate being

formed from α -ketoglutarate and carbon dioxide. Ochoa, Mehler & Kornberg (72) have recently presented evidence, however, that malate may be formed from pyruvate and carbon dioxide without the intermediary formation of free oxaloacetate, by direct reduction with reduced triphosphopyridine nucleotide (TPNH₂). They obtained a protein fraction from pigeon liver which could catalyze the reversible reaction,



This reaction was followed spectrophotometrically. Oxaloacetate could not oxidize TPNH₂, but was, however, decarboxylated by the enzyme. ATP was reported to be without effect on the reaction. To explain previous findings of Utter & Wood that ATP stimulated the exchange of isotopic carbonate with the β -carboxyl carbon of oxaloacetic acid, it seemed reasonable to suppose that ATP caused the formation of TPN, a reaction which could actually be demonstrated in pigeon liver (73). Direct tests of the effect of TPN on the exchange reaction (74, 75) revealed, however, that this could not be the explanation, since TPN could not substitute for ATP. It was found, however, that TPN could stimulate the decarboxylation of oxaloacetic acid—a curious effect for a cofactor thought to be concerned only with oxidoreductions. Future work will undoubtedly reveal the actual relations of these apparently contradictory facts. For the present, the point which deserves emphasis is the intimate association, possibly on the same enzyme molecule, of the decarboxylation and the TPN-mediated oxidoreduction, as demonstrated by Ochoa *et al.* (72).

Evidence is at hand that a similar situation may obtain in plant tissues. Vennesland & Felsner (76) have demonstrated the occurrence of oxaloacetic carboxylase in a number of plant preparations. The enzyme from parsley root was chosen for further study because of its stability and similarity in several respects to the animal enzyme. Gollub & Vennesland (77), and Vennesland, Ceithaml & Gollub (78) have shown that the active parsley root preparation, which decarboxylates oxaloacetate in the presence of manganese ion as a cofactor, contains an oxalosuccinic carboxylase as well as an oxaloacetic carboxylase and that both are reversible. Furthermore, a malic dehydrogenase and an isocitric dehydrogenase active with TPN are present in the same preparation. The reversibility of both systems may therefore be demonstrated spectrophotometrically as well as with the aid of isotopic carbon.

The finding that plants can fix carbon dioxide by these routes may be indicative of a basic similarity in the metabolic mechanisms whereby carbohydrates and the di- and tricarboxylic acids are interconverted in both plants and animals. It has been postulated that the type of carbon dioxide fixations demonstrated here are intimately associated with the carbon dioxide assimilating mechanisms of green plants, which operate during photosynthesis (79, 80). A few facts which seem, to the author, to have some bearing on this point, may therefore be summarized here.

(a) The two demonstrated fixation reactions, taken in conjunction with the tricarboxylic acid cycle as it operates in animal tissues, are not able to cause any "net" fixation of carbon dioxide.

(b) There is no evidence whatever that the two carboxylation reactions under discussion are the only mechanisms whereby plants fix carbon dioxide.

(c) Studies with photosynthesizing systems have given results which can be interpreted in part by fixation in the di- and tricarboxylic acids (80, 81, 82). However, though the nature of all the initial fixation reactions is not yet known, the weight of evidence indicates that during illumination, mechanisms quite different from those described are quantitatively more important (81, 83).

A very interesting development in our knowledge of the mechanism of carbon dioxide fixation into di- and tricarboxylic acids concerns the recent accumulation of indirect evidence that biotin participates in the fixation reaction. Most of this work has been done with microorganisms. For earlier work on the function of biotin, the reader is referred to the review of Hertz (84). Present developments were foreshadowed by the demonstration of Koser *et al.*, in 1942, that the biotin requirements of a yeast were reduced by aspartic acid. Lyman *et al.* (85) later demonstrated that carbon dioxide was required for aspartic acid synthesis by some lactobacilli. Stokes *et al.* (86) showed that several lactic acid bacteria could grow with suboptimal amounts of biotin if aspartic acid were supplied. Lardy *et al.* (87, 88) then demonstrated that oxaloacetic acid can promote growth of *Lactobacillus arabinosus* on media deficient in biotin and aspartic acid, and that bicarbonate stimulates growth in the presence of biotin, but not in a biotin-low medium. Shive & Rogers (89) concluded from application of an "inhibition analysis" that biotin was concerned in the biosynthesis of oxaloacetic and α -ketoglutaric acid by microorganisms and quote

hitherto unpublished results of Garrison & Eakin, which indicate that biotin functions in yeast in the carboxylation of pyruvic acid to form oxaloacetic acid. Lichstein & Umbreit (90) report success in demonstrating that addition of biotin to certain *E. coli* preparations increases the rate of carbon dioxide evolution obtained from added oxaloacetate. Ochoa *et al.* (91, 92) have demonstrated that tissues of biotin deficient turkeys were deficient in oxaloacetic carboxylase but were unable to demonstrate the presence of biotin in the purified enzyme. It seems that there is no direct evidence yet that biotin is a constituent of the enzymes responsible for carbon dioxide fixation, though there is strong evidence that biotin is involved in the reaction.

Further work on carbon dioxide fixation involves the demonstration, by Delluva & Wilson (93), that isotopic carbon dioxide administered to the intact animal is incorporated into the aspartic and glutamic carboxyl groups of the proteins. Anfinsen *et al.* (94) have demonstrated a similar phenomenon in liver slices. Kritzmann (95) has presented evidence that liver and kidney extracts synthesize alanine from pyruvate and ammonia by a route which involves first, a synthesis of oxaloacetate from pyruvate and carbon dioxide, then a reductive amination of oxaloacetate to form aspartate, and finally, a transamination between aspartate and pyruvate to yield alanine and regenerate oxaloacetate.

PYRUVATE OXIDATION AND THE TRICARBOXYLIC ACID CYCLE

Pyruvate, the end product of aerobic glycolysis, occupies a pivotal position in carbohydrate metabolism of animals, since the fate of the carbon chain from this stage on may involve transformation to alanine and so to protein, carboxylation to a dicarboxylic acid, transformation to fat, or complete oxidation by way of the tricarboxylic acid cycle.² The conversion of pyruvate to fat, and the oxidation of pyruvate, are both thought to involve a preliminary oxidative decarboxylation of the three-carbon chain, with the production of a two-carbon unit.³ The precise nature of this two-carbon unit is as much a subject of speculation today, as it was

² It is the author's opinion that the weight of experimental evidence at present supports such a view, at least in regard to muscle, kidney and liver. For opposing points of view, see p. 241.

³ There is actually no evidence to indicate whether the carboxyl group of pyruvate is removed before or after condensation with a four-carbon dicarboxylic acid.

two years ago (96). It is regarded at present as a kind of activated acetyl (97) or ketene (98), a substance which may exist only in combined form, and which can probably be converted to and formed from acetate under some, but not under all circumstances. The possibility exists that there may be several types of such two-carbon units.

The conversion of pyruvate to acetate has been demonstrated in a number of different types of preparations. Recent work includes that of Stumpf *et al.* (99), who have described a tissue fraction which can catalyze the oxidative decarboxylation of both pyruvic acid and of α -ketoglutaric acid. Diphosphothiamine is required. The diacetyl mutase described by Green *et al.* (100) is also of interest in this connection. This enzyme catalyzes the dismutation of two moles of diacetyl to form two moles of acetic acid and one mole of acetoin. Kalnitsky & Barron (101) have studied the effects of fluoroacetate on the metabolism of yeast and bacteria, and Bartlett & Barron (102) have studied the action of this inhibitor on enzymes and tissue metabolism. They report that fluoroacetate causes an inhibition of pyruvate oxidation with accumulation of acetate. A number of bacterial preparations which convert pyruvate to acetate have also been described (103, 104, 105).

Though bacteria may form pyruvate by addition of carbon dioxide to a two-carbon unit, there is no evidence that such a reaction occurs in animal tissues. Here the decarboxylation of pyruvate seems to be irreversible. Once the two-carbon unit is formed it may be converted to fat, but fat which may form two-carbon units cannot be reconverted to carbohydrate.⁴ Deuel & Morehouse (106) have recently reviewed the interrelation of carbohydrate and fat metabolism and summarized evidence for and against this somewhat controversial point.

The metabolism of acetate *per se* is outside the scope of this review. Because of the significance of the active two-carbon unit in carbohydrate metabolism it is not possible, however, to exclude all aspects of acetate metabolism. Lipmann (97) has reviewed the properties of acetyl phosphate and discussed the role of this substance in metabolism. Bloch (107) has also recently published a review of acetate metabolism in the animal body and discussed the

⁴ The possibility that acetate may form carbohydrate by way of condensation to succinate need not be considered until proof of the existence of such a reaction in animal tissues is provided.

phenomena of acetylation as well as fat formation in detail. Other valuable contributions to this subject include the studies of Kinunen (108) on the relation between acetylation of sulfonamides and thiamine balance, and the investigations of Smyth (109) on the rate and site of acetate oxidation in the intact animal.

One of the most interesting developments in recent work on acetylation mechanisms has provided evidence for the site of the chemical action of pantothenic acid. Lipmann & Kaplan (110) have demonstrated the existence of a new cofactor, coenzyme A, which is required for the enzymic acetylation of sulfanilamide by acetate with adenylypyrophosphate as an energy donor. This cofactor is apparently identical with that required for the analogous acetylation of choline. Lipmann *et al.* (111) have purified coenzyme A seven hundredfold and found that the product contains pantothenic acid and that the activity of the preparation parallels the pantothenic acid content during the course of the purification.

Some years previously, Dorfman *et al.* (112) had shown that pantothenic acid is necessary for pyruvate oxidation by the bacterium *Proteus morganii*, and Hills (113) had demonstrated that the pantothenate effect applies to the second or acetate phase of pyruvate oxidation. Novelli & Lipmann (114) have confirmed these findings and have shown further that the bacteria convert added pantothenic acid into coenzyme A and that the increase in coenzyme A parallels the respiratory stimulation by pantothenate. If future findings substantiate the supposition that pantothenic acid derivatives may function in the oxidation of two-carbon units generally, then it would seem that coenzyme A or similar substances must play a vital role far more significant than as an activator of acetylations. Recent experiments with *E. coli* by Shive *et al.* (115) have some bearing on this point, as well as on the mode of oxidation of the two-carbon unit. These authors used cysteic acid to inhibit the conversion of aspartic acid to β -alanine by the bacteria. Under such circumstances *E. coli* cannot synthesize pantothenic acid, and addition of this factor is then required for growth. The requirement for pantothenic acid (in the presence of cysteic acid) can be decreased by the addition of citric, *cis*aconitic, and α -ketoglutaric acids, but not by oxaloacetate and pyruvate. The authors conclude that organisms deficient in pantothenic acid cannot synthesize the tricarboxylic acids or α -ketoglutaric acid out of their precursors, and suggest that pantothenic acid functions in

the formation of the hypothetical "active" acetyl radical which is believed to condense with oxaloacetate to form the tricarboxylic acids. Such an interpretation is in harmony with present hypotheses regarding the mode of oxidation of two-carbon units.

Although Weinhouse *et al.* (116) have studied labeled acetate and acetoacetate oxidation in liver and kidney and found that the citrate formed contained isotopic carbon only from acetoacetate but not from acetate, this is not regarded as evidence against the view that both pyruvate and acetate are oxidized by way of the tricarboxylic acid cycle. Citrate is now regarded as a side product of the cycle. Weinhouse & Millington (117) have recently studied glucose and acetate oxidation in yeast and concluded that the mechanism of oxidation of pyruvate and acetate are similar to those which obtain in animal tissues. Evidence that Krebs' cycle operates in the intact animal has been afforded by the work of Wood and his collaborators (118). These results represent a systematic study of the distribution, in newly formed liver glycogen, of isotopic carbon administered in the form of a number of glycogenic and ketogenic substances. The results can be explained fully by postulating that the reactions of the tricarboxylic acid cycle operate *in vivo* in the manner deduced from *in vitro* studies. Perhaps it is still necessary to point out that the appearance in glycogen of labeled carbon derived from a fatty acid does not indicate a net formation of carbohydrate from fat. The reader is referred to the original papers for details regarding the manner in which the operation of the cycle as a common pathway for carbohydrate and fat oxidation leads to a redistribution of carbon in the component members of the system.

Some interesting general considerations regarding the functional significance of cyclic processes in living matter have been published by Krebs (119), who has emphasized that reversibility of a series of biological transformations is often cyclic rather than direct. Krebs has pointed out that it is the irreversible reactions in a cycle that determine the direction in which the cycle must operate, and such control of direction is undoubtedly necessary for proper maintenance of the living system. Two main reactions of the tricarboxylic acid cycle: the initial condensation to a tricarboxylic acid and the oxidative decarboxylation of α -ketoglutarate have thus far not been shown by unequivocal evidence to be reversible in animal tissues. Recent claims that the first of these may

be reversed under certain circumstances (120) will therefore be of considerable interest if substantiated.

The oxidation of pyruvate in dialyzed dispersions of beef heart and brain have been investigated by Gibson & Long (121) and by Long (122). Phosphate, adenine nucleotide, magnesium ion and fumarate are necessary additions for these systems. The authors conclude that fumarate acts catalytically in ox heart (121) but that in the brain preparation (122) fumarate, though it increases oxygen consumption in the presence of pyruvate, does not increase the rate of pyruvate removal. It is implied that the tricarboxylic acid cycle may not operate in brain. Perhaps the interpretation of the significance of such results requires more information about the stability of the dicarboxylic acids in such systems. Though the tricarboxylic acid cycle provides for the regeneration of oxaloacetate, little is known about the relative tendency of this unstable compound to decompose in various preparations after being regenerated but before being reused.

Peters (123) has reviewed the role of thiamine in pyruvate metabolism, and also summarized recent British work on the mode of action of lewisite (124 to 127). This substance is said to produce symptoms similar to B₁ deficiency and is thought to inhibit pyruvate oxidation by combining with sulfhydryl groups necessary for the enzyme activity. BAL (2,3-dimercaptopropanol) protects the enzyme by competing for arsenic derivatives.

Breusch & Kara (128) have presented evidence that oxaloacetate reduction occurs more rapidly than citrate turnover and Breusch & Tulus (129) have classified a number of tissue breis with regard to their ability to cause removal of citrate and reduction of oxaloacetate. The close association of these activities is striking.

A number of enzyme studies of related interest have appeared. Among them may be mentioned the work of Schneider (130) and of Hogeboom *et al.* (131) on the distribution within the cell of succinic dehydrogenase, cytochrome oxidase, and related activities. The latter authors have made a preliminary report of the preparation of a soluble succinic dehydrogenase. Furthermore, Stoppani (132) has described a soluble factor from liver which links succinic dehydrogenase to the cytochrome system. Several studies of the ability of succinic dehydrogenase to labilize the hydrogen attached to the methylene groups of the substrate have also been made (133, 134, 135). Another enzyme study of great

interest is the isolation by Bach, Dixon & Zervas (136) of a lactic dehydrogenase from yeast which apparently contains cytochrome- b_2 within the molecule. No pyridine nucleotide or flavin is required as a mediator.

The role of the components of the Krebs cycle in nitrogen metabolism is outside the scope of this paper, but the reader is referred to a review of the subject by Braunshtein (137). Recent reports of Greenstein and his associates that pyruvate may function in the desamidation of glutamine (138, 139, 140) may be of interest in this connection, as may McIlwain's (141) observation that certain streptococci preparations hydrolyze glutamine only during glycolysis. The ability of pyruvate to accelerate purine oxidations has also been described (142).

The possibility that tissues may oxidize hexoses directly by a successive removal of one carbon atom at a time continues to receive attention (143, 144). Schlenk & Waldvogel (145, 146) have described the conversion of pentose from guanosine into Robison ester by a liver enzyme. The authors favor the interpretation, however that the pentose does not add on one carbon to form a hexose but that the five-carbon chain is split into smaller units and that two three-carbon chains then recombine.

MISCELLANEOUS

A number of substances have been tested for ability to act as glycogen precursors. Wiebelhaus, Bethel & Lardy (147) found that *meso*-inositol caused no increase in liver glycogen in fasted rats. Since this substance did function antiketogenically, failure to cause glycogen deposition was thought to be associated with very slow absorption from the intestines. Carr *et al.* (148) found that sorbitol is a glycogen precursor but sorbitan is not. Schofield & Lewis (149, 150) have made a comparative study of alanine, β -alanine, serine and isoserine with regard to their rate of absorption from the gastrointestinal tract and their ability to serve as liver glycogen precursors. The rate of absorption was in the order D- and DL-alanine > DL-serine > L-alanine > β -alanine > DL-isoserine. The ability to form liver glycogen followed the same sequence, except that β -alanine was not glyconeogenic.

Nieft & Deuel (151) have observed that dietary fat inhibits intestinal absorption of galactose. Ershoff (152, 153) has described

the ability of galactose to cause paralysis in rats receiving certain purified rations and noted a curative effect of yeast. Wolfrom *et al.* (154) have isolated from beef lung a polysaccharide containing no sugar except D-galactose. Mann and his collaborators (155, 156) have continued their studies of the occurrence and function of seminal fructose. Fructose has been shown to be the chief reducing sugar of the semen and serves as a readily fermentable substrate for the glycolytic system of the spermatozoa. The seminal vesicles are the main source (156) and fructose formation begins before spermatogenesis (157). The fructose content of semen drops rapidly after castration and shows a rapid rise on administration of testosterone (158). Bacon & Bell (159) have made a quantitative study of the fructose content of foetal and maternal blood of sheep throughout the period of gestation.

Lipscomb & Crandall (160) have measured the glucose output of the liver of normal, unanesthetized dogs and found it to average 122 mg. per kilo per hr. Himwich & Himwich (161, 162) have studied blood pyruvate in normal dogs and in diabetic dogs and patients. A pyruvate cycle resembling that of lactic acid, between liver on one hand, and muscle and intestines on the other, was observed. In the resting diabetic, injected glucose did not produce the rise in blood pyruvate observed in the normal. The results support the view that insulin facilitates the phosphorylation of glucose. The utilization of glucose by the gastrointestinal tract of normal and diabetic dogs has been studied (163), as well as the effect of fasting on glucose tolerance (164, 165). Observations are available on carbohydrate tolerance tests in swine (166), blood sugar values in mice (167) and chicks (168), seasonal variations in liver glycogen of the frog, and the effect of the hypophysis thereon (169, 170), the maintenance of rat liver glycogen after feeding individual amino acids (171), and the effect of the fat content of the diet on blood sugar (172). Banerjee & Ghosh (173, 174) have studied carbohydrate metabolism of scorbutic guinea pigs and suggested that the disturbances noted are due to a deficiency of insulin secretion. The mechanism of phlorhizin glycosuria (175, 176) and the effect of mustard gas on carbohydrate metabolism *in vivo* (177) have been examined. A number of studies relating to carbohydrate metabolism of brain (178 to 183) have also appeared, as well as a report that glutamic acid can cause an increase in blood sugar during insulin shock (184).

ALLOXAN, HORMONES AND DIABETES

Because of space limitations and the volume of literature in this field, the author has been forced to omit a large body of interesting publications. The following listing is a partial substitute for complete omission of these topics.

Riddle and his associates (185) have published a series of papers which represent seven years of work on regulatory mechanisms of carbohydrate and fat metabolism in birds and mammals. The similarities between these two classes of vertebrates were found to be more striking than the differences. Several studies of the diabetogenic action of substances related to alloxan (186, 187, 188) have appeared, notably that of Brückmann & Wertheimer (189), who have tested twenty-nine such compounds for their ability to induce diabetes in rats. An intact pyrimidine nucleus was found necessary for diabetogenic action; and the activity could not be correlated with ability to cause deamination and decarboxylation of amino acids, or with ability to oxidize sulfhydryl groups. Gaarenstroom (190), Iversen (191), and deOya & Covián (192) have studied the influence of the hypophysis in alloxan diabetes, and Houssay *et al.* (193) have summarized their studies on the influence of the thyroid on alloxan and pancreatic diabetes in the rat. Poulsen (194) and Goldner & Gomori (195) have presented studies on the mode of action of alloxan. Several investigations of the relation of alloxan action to phosphatase activity have appeared (196, 197, 198). The ability of BAL (2,3-dimercaptopropanol) to protect rats against alloxan diabetes has been described (199).

Bouckaert & de Duve (200) have reviewed the subject of the action of insulin. A number of interesting papers on the chemistry of insulin (201 to 206) and on the effect of insulin and carbohydrate on amino acid levels of blood and urine (207 to 211) have appeared. Stoppani (212, 213) has investigated the relation between the di- and tricarboxylic acids, blood phosphates, and the diabetic syndrome. The average hepatic glucose output in the diabetic dog has been found to be the same as in the normal (214). Studies have appeared on creatinuria in diabetes (215), and of the inorganic constituents of the blood during diabetic acidoses (216, 217). The action of biologically and chemically pure growth and adrenocorticotrophic hormone on carbohydrate metabolism has been investigated (218, 219, 220). Studies are also available of the

inhibition by insulin of the liver fat deposition caused by anterior pituitary extract (221); of the glycosuria induced by 17-hydroxycorticosterone and corticosterone in rats force-fed carbohydrate (222); of the increase in blood sugar induced by adrenal cortex extract and insulin in eviscerated rats (223); of the role of the adrenals in mediating lymphocytopenia due to glucose administration (224); and of the impaired pituitary gonadotrophic function in diabetes (225).

PREPARATIONS AND METHODS

Methods have been described for the preparation of phosphomalic acid (226), esters of lactic acid with glycerol and fructose (227), lithium acetyl phosphate (228), and diphosphopyridine nucleotide (229, 230, 231). Procedures for determining glycogen have been studied (232, 233), and two investigations of blood glycogen have appeared (234, 235). Breusch & Tulus (236) have made a detailed study of the specificity of the pentabromacetone method for citrate; modifications of the citrate method have been described (237, 238, 239); and the occurrence of citrate in fowl's blood (240), in human thyroid (241), and in human saliva (242) have been studied. The assay of insulin has been investigated (243, 244). A modified procedure for serum amylase (245), a method for blood inositol (246), a procedure for determining pentose in the presence of large quantities of glucose (247) and a method of differentiating ribose-3-phosphate and ribose-5-phosphate (248) have been described. Determinations of adenosine, adenosine-5-phosphate, adenosine-3-phosphate, ATP and ADP by a combination of enzymatic spectrophotometric methods (249, 250) have also been presented. Other procedures which have appeared within the last year include a color reaction for hexuronic acids (251), a method for glucuronic acid (252), several methods for blood sugar (253, 254, 255), and the application of partition chromatography to the estimation of reducing sugars (256, 257) and organic acids (258).

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LIPID METABOLISM

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ABSORPTION, DIGESTION, AND EXCRETION

A much needed appraisal of the present status of the mechanism of fat digestion and absorption has come from Schmidt-Nielsen (1). In addition he investigated two phases of this problem, viz., the significance of phosphorylation of fat in the small intestine and the role of soaps in fat absorption. The former was studied in rats with radioactive phosphorus. Although he found an increase in the specific activity of phospholipid phosphorus of the rat's small intestine during fat absorption, he calls attention to the fact that the increase was insufficient to account for the conversion of the absorbed fat to phospholipid, unless the phosphorylation process be limited to the epithelial cells of the small intestine. The older concept that fats may be absorbed in the form of soaps has been given new meaning. At the pH of the small intestine he found that a considerable proportion of the total fat may exist as soaps. He also furnished evidence that bile may promote the fatty acid-soap equilibration.

According to Frazer (2, 3) ingested triglycerides, at least the bulk thereof, are finely dispersed in the small intestine and are then absorbed as unhydrolyzed particles directly into the lacteals. Proof of particulate absorption rests in part upon the experiments of Frazer *et al.* (4), in which it was demonstrated that a paraffin emulsion of particle size less than 0.5μ is absorbed from the intestine as readily as an olive-oil emulsion of similar dispersion. This support of the partition hypothesis is now questioned by Lundbaek & Maaløe (5). In a careful study they were not able to demonstrate the absorption of paraffin emulsions of particle size as low as 0.3 micron.

A unique case of hepatic cirrhosis in which an enlarged abdominal vein communicated with the portal venous system was studied by Sherlock & Walshe (6). They estimated that the anastomotic channel carried from a quarter to a half of the portal venous blood directly into the right great saphenous vein. Yet no rise in the fat content of this abdominal vein was observed when 75 gm. of butter were introduced into the duodenum.

Crockett & Deuel (7) report that hydrogenated lard with an m.p. of 55° is more slowly absorbed by the rat than fats with m.p. ranging from 34° to 48°, namely margarine, crisco, prime steam lard and bland steam lard. The amount of fat appearing in the feces as soaps increased sharply when the melting point of lard was raised from 48° to 55° by hydrogenation. It was concluded that the increased fat excretion observed with higher-melting fats is due to the rat's inability to absorb the larger amounts of palmitate and stearate formed by hydrogenation, rather than to a failure of lipolysis. Similar results were obtained by Augur, Rollman & Deuel (8) when cotton seed oil hydrogenated to different degrees was fed to rats. These investigators also noted that the addition of crude lecithin to the diet increased the absorption of fats, particularly the more highly saturated ones.

From a careful analysis of hitherto published data, Mattil (9) concludes that

the primary factor which limits the digestibility of fats is, without doubt, the amount of saturated fatty acids present, and the degree of limitation increases with chain length.

He has shown that a positive correlation exists between the digestibilities of fats found in human adults and those of corresponding fats found in albino rats.

The question of the metabolic significance of fecal fat has been reopened by Wollaeger *et al.* (10). They cite their own carefully obtained findings, as well as those of others, to show that the quantity of fat lost in the feces of normal subjects is influenced largely by the amount of fat ingested. They therefore suggest that unabsorbed dietary fat accounts for a large portion of fecal fat. Since the amount of fecal fat is influenced by the amount and kind of fat ingested, Wollaeger *et al.* call attention to the need of standardization of the fat in test diets for use in the detection of abnormal absorption or abnormal fecal fat excretion (steatorrhea).

According to Phillipson (11) volatile fatty acids consisting largely of acetic and propionic acids are found in the large intestine of the dog. Their absorption is suggested by his finding a higher concentration of such fatty acids in the blood leaving the large intestine than in any other venous or arterial blood sample.

PARENTERAL ADMINISTRATION OF FAT

The direct administration of nutrients into the blood stream has been actively studied during the past few years. In order to

provide sufficient calories in reasonable volumes of administered fluid, investigators have turned to a study of the parenteral administration of fats. The dangers inherent in the use of impure lecithin for stabilizing fat emulsions were pointed out by Ashby (12). This worker found that commercial grades of lecithins were toxic to rabbits when administered in doses of 1.75 gm. per kg. A freshly prepared egg lecithin that was not toxic at twice this dosage level became so on standing in contact with air. Judging from nitrogen excretion and quality of depot fat, Dunham & Brunswick (13) could not conclude that intravenously injected fat is utilized. McKibbin, Ferry & Stare (14), on the other hand, were able to demonstrate that the daily intravenous administration of coconut oil emulsions to dogs that received an oral ration inadequate in calories prevented weight loss and produced an increase in nitrogen retention. In two dogs they could not account for 43 and 63 per cent of the infused coconut oil and therefore concluded that fat given intravenously is utilized for energy purposes. An emulsion has been used clinically by Shafiroff & Frank (15). No evidence was advanced to show utilization of the fat although the patients were maintained satisfactorily.

Both Dunham & Brunswick and McKibbin *et al.* found toxic changes after the prolonged injection of fat. Changes in the reticulo-endothelial system and impairment of liver function showed that the particle size of the infused fat must be carefully controlled. Shafiroff & Frank found no evidence of lipoid granulomatosis in dogs after thirty-five daily injections of a coconut-oil emulsion.

INFLUENCE OF ENDOCRINES ON LIPID METABOLISM

Pituitary.—The presence of a factor or factors in crude extracts of the anterior pituitary gland capable of increasing the fat content of the liver was confirmed by Weil & Stetten (16) and Riddle & Opdyke (17). Attempts to identify the factors were not successful (18, 19). The claim of Riddle & Opdyke that injections of insulin increased the fat content of the liver of the pigeon is surprising in view of Campbell's finding (20) that the increase in liver fat produced by anterior pituitary extracts in fasting rats, mice, and guinea pigs can be partially inhibited by insulin.

According to Weil & Stetten (16) the urine of the fasted rabbit, but not of the fed, contains a substance which when injected into mice increases the fat content of their livers. Apparently these

workers believe that fat mobilization is under control of the anterior pituitary gland, for they point to this gland as a possible site of origin of the urinary factor.

Reiss found that the fat content of the skin is reduced by the injection of anterior pituitary fractions containing lactogenic hormone (21). Thyroid administration facilitated this effect of the hormone.

It would appear that little more is to be gained by further study with crude extracts. Indeed, the work so far presented fails to answer the question whether anterior pituitary tissue contains a specific hormone (other than thyrotropic) which has to do with the control of fat metabolism. This problem can be unravelled only by the use of pure hormones which have been identified as individual substances.

Hypothalamus.—The obesity that results from experimentally induced hypothalamic lesions is an amazing biochemical phenomenon. Rats develop voracious appetites immediately after the hypothalamic operation, and an adult female rat may increase its body weight by as much as 15 per cent within the first twenty-four hours if fed *ad libitum* (22). It is now clearly established that this type of obesity does not involve the anterior lobe of the pituitary, for it can be made to occur in animals deprived of this lobe. A comprehensive review of the mechanism of the development of hypothalamic obesity has been presented by Brobeck (22). Various phases of the metabolism of the hypothalamic-obese rat (eating habits, activity, effects of limitation of food intake, digestion, and respiratory quotients) have been dealt with in a series of papers by Brooks and his associates (23 to 27).

Adrenal.—The isolation from the adrenal cortex of a new fat factor was reported by Hartman, Brownell & Thatcher (28). Its administration brings about deposition of fat in the liver of the fasted adrenalectomized animal. Crystalline corticosterone, 11-dehydrocorticosterone, and 11-dehydro-17-hydroxycorticosterone do not influence the liver-fat content in this animal preparation. The capacity of cortical cells to produce this fat factor has also been studied (29).

In confirmation of earlier findings of MacKay & Carne (30), Berman *et al.* (31) report that adrenalectomy interferes with the deposition of excessive amounts of fat that occurs in the liver remnant twenty-four hours after partial hepatectomy.

Estrogens.—Although the concentrations of the various lipids

in the blood and liver of the male and immature female bird do not differ greatly from those in other species, such as man and dog, avian lipid metabolism differs from mammalian in being under the control of ovarian hormones. In the bird actively engaged in egg-laying, total lipids of the blood may rise as high as 5000 mg. per 100 cc. as compared with about 500 mg. in the nonlaying state. This rise in lipids can be reproduced in the immature female bird by the stimulation of ovarian activity or in the male bird, as well as in the immature female bird, by the injection of crystalline estrogenic compounds. In view of the close relation between birds and reptiles in their phylogeny and ovulation, it is of interest to record that increased ovarian activity in the female turtle, as judged by the presence in the ovary of numerous large ova, is also accompanied by a rise in the concentration of lipids in the blood (32). Since the ova of birds and turtles are rich in fat, it is reasonable to believe that the rise in blood lipids during ovarian activity represents a mechanism that makes possible a rapid mobilization of fat for use in yolk formation.

In confirmation of earlier work, Riddle, Senum & Rauch (33) report that estrone, dihydroestrone, and estradiol benzoate, when injected or implanted as pellets, increase the concentration of plasma fat in pigeons of both sexes. They have shown for the first time, however, that the lipemia induced in the bird by these estrogens does not require the presence of the pituitary gland, the adrenals, pancreas, or parathyroids.

Lorenz & Bachman (34) discuss the value of various estrogens for poultry fattening. The superiority of orally administered dianisylhexene over diethylstilbestrol is pointed out. Dienestrol acetate was found to be as effective as dianisylhexene. Since estrogenic fattening occurred in the absence of a lipemia, these investigators argue that the estrogen-induced lipemia represents a piling up of fat in the blood faster than it can be absorbed into the depots.

According to György *et al.* (35) the lipotropic action of methionine in intact and castrated female rats fed a high fat-low protein diet can be greatly augmented by the oral administration of 30 μ g. of estrone daily. In these rats the hormone itself was also found to have a small but definite lipotropic effect.

NUTRITIONAL ASPECTS OF LIPIDS

The superiority of diets containing liberal amounts of fat has been established for the rat by Deuel and his associates (36, 37,

38). In their studies they employed such criteria as growth, physical capacity, reproduction, lactation, etc. Their findings indicate that fat not only serves as a storage material in the physiological economy of the animal body, but actually participates in vital processes as a component in what they term the metabolic machinery. The better performance observed with diets richer in fat, according to their view, results from the fact that the necessity of converting carbohydrate to fat is in part circumvented.

A review of some of the literature dealing with the influence of fat enrichment of the diet upon tumor formation has been made by Tannenbaum (39) and by Opie (40). A general conclusion for all tumors cannot be drawn. Thus a fat-enriched diet enhances formation of spontaneous mammary carcinoma in mice but has no effect on the formation of lung adenoma or upon the incidence of spontaneous and induced leukemias. As might be expected, the fat content of the diet has a profound effect on the incidence of liver tumors produced by butter yellow (*p*-dimethylaminoazobenzene). Opie reports that when the fat content of the diet was 43 per cent tumors appeared in 66 to 69 per cent of the animals; with a fat content of 59 per cent, the frequency of tumors was 82 per cent; and with 87 per cent of fat in the diet, all animals had tumors.

Nieft & Deuel (41) have shown that the rate of intestinal absorption of galactose varies inversely with the percentage of fat in the diet. The importance of this finding lies in the fact that it helps to explain the occurrence of galactosuria in animals fed solely on liquid skim milk and the failure of its occurrence when whole milk is fed or when fat is added to the skim-milk diet.

Artom & Fishman (42) found that in rats fed a low-protein diet the substitution of equal parts of lactose and dextrin for equal parts of sucrose and dextrin resulted in a lower neutral-fat content in the liver. Similar low levels of neutral fat were found when the sole carbohydrate source was dextrin, lactose, or galactose, but not when it was glucose, sucrose, or maltose. Slight increases in the lecithin levels usually accompanied the lower amounts of neutral fat. The lipotropic action of choline was greater when lactose was present in the diet.

It is well known that the fatty acid composition of phospholipids and of liver triglycerides is determined in part by the nature of the fat in the diet. Sinclair & Chipman (43) have now shown

that this holds also for the cholesterol-ester fatty acids deposited in the liver.

The thesis that essential fatty acids are among the nutritional factors concerned with maintenance of the normal integrity of the skin has again been pointed out by Hansen *et al.* (44). They found that among 171 patients suffering from an eczematous condition of the skin 80 per cent of infants under two years of age, 75 per cent of children between two and fifteen years, and slightly over 50 per cent of adults had iodine numbers for their serum fatty acids below those of normal subjects. The administration of fats rich in unsaturated fatty acids improved the clinical condition of the skin and increased the degree of unsaturation of the serum fatty acids. A summary of the role of essential fatty acids in nutrition, with particular reference to human beings, will be found in a report prepared by Hansen & Burr (45).

Bernhard & Bodur (46) fed pregnant rats a fat-free diet during the latter part of gestation. Although linoleic acid was still present in the tissues of the mothers, none was found in their young.

Loeb & Burr (47) failed to find a sex difference in the amounts of total body-fat in rats fed a normal diet. Only when rats were fed a diet high in saturated fats but deficient in essential fatty acids did a sex difference in the amounts of body fat appear. In the latter instance, storage of body fat was impaired in both sexes but less severely in the females than in the males. Despite impaired storage, however, evidence was obtained that an exchange had occurred between dietary fat and body fat.

PHOSPHOLIPIDS

The nature of plasma phospholipids and their metabolic significance.—It was demonstrated by Taurog *et al.* (48) that nearly all of the phospholipids in plasma of man and dog contain choline. This indicates that plasma phospholipids of these two species are composed mainly of lecithins and sphingomyelins. Confirmation of this finding in the case of man has come from Hack (49) and Sinclair (50). The former reports that about 95 per cent of the phospholipids in the serum of normal human subjects are choline-containing. Sinclair measured the choline-to-phosphorus ratios of serum phospholipids in a variety of animals. For man and beef the ratios were 0.99 to 1.00. Thus the plasma or serum of man, dog, and beef appears to contain no cephalins. This is not the case,

however, in the bird, for significant amounts of cephalins have been found in turkey sera (50) and in the plasma of the domestic fowl (51).

The current view of the function of plasma phospholipids has been questioned by Entenman *et al.* (52). These workers found that the concentration of phospholipids is not appreciably decreased by excluding the liver from the circulation. Thus in one experiment 385 and 388 mg. of phospholipids per 100 cc. of plasma were found respectively immediately before and nine hours after the liver had been excluded. Since plasma phospholipids are synthesized mainly in the liver, this constancy in the level of plasma phospholipids in the liverless dog means that in the latter the utilization of plasma phospholipids has practically ceased. Further evidence for this concept was obtained by comparing the rates at which intravenously injected labeled phospholipids (with radioactive phosphorus) disappear from the plasma of normal and liverless dogs. Plasma phospholipids were completely turned over in six to ten hours in normal dogs weighing seven to eighteen kilos; by depriving these dogs of their livers, the time required for complete turnover was prolonged to thirty-three to one hundred and sixty hours. These findings suggest that the liver is the principal tissue in the body concerned with not only the synthesis and supply of plasma phospholipids but also with their removal.

The lipemia that occurs in the fasted mouse has received further attention from Hodge *et al.* (53). It is of interest to recall that in the three-month-old mouse utilizable carcass lipids disappear in two days of fasting. These workers observed a progressive increase in blood phospholipids for the first four days. Levels as high as 450 mg. per cent were found on the fourth day of fasting as compared with 275 mg. in the nonfasted state. For the reasons presented above, it is hardly likely that these increased amounts of plasma phospholipids are concerned with transport of fat to the periphery. The possibility should be considered that plasma phospholipids like their precursors, liver phospholipids, represent a stage in the oxidation of long-chain fatty acids by the liver.

Lymph.—Methods for obtaining thoracic duct lymph from dogs and rats have been greatly improved by Bollman, Flock and their associates (54, 55). These workers report that during fasting the concentration of phospholipids is higher in plasma than in thoracic duct lymph in the case of the dog but lower in the rat.

The feeding of a fat-free meal failed to increase the phospholipid concentration of this lymph in both dog and rat. A meal containing either neutral fat or free fatty acids (but no phospholipids) did, however, produce an increase in the phospholipid content of thoracic duct lymph of the dog. In dogs injected with radioactive phosphorus, they were able to demonstrate that the intestine as well as the liver added new phospholipid to lymph. The contribution by the intestine was increased after fat feeding.

Methods.—Recent developments in the separation of the various phospholipids should make possible a more effective study of the metabolism of cephalin, lecithin, and sphingomyelin. Non-choline (cephalins) and choline-containing phospholipids (lecithins and sphingomyelin) can be quantitatively separated by the use of magnesium oxide as described by Taurog *et al.* (56). The lecithin and sphingomyelin contents of the choline-containing fraction can then be determined by the method of Schmidt *et al.* (57). The latter depends upon the finding that lecithins and cephalins are quantitatively transformed into acid-soluble phosphorus compounds by treatment with normal potassium hydroxide at 37° for twenty-four hours. Under the same conditions sphingomyelin does not form acid-soluble phosphorus compounds. In addition, Hack (58) found that such treatment with potassium hydroxide liberated choline quantitatively from lecithin but not from sphingomyelin. Thus by measuring (a) total phosphorus of a lipid extract and (b) the choline liberated and the acid-soluble phosphorus formed by treatment of the extract with alkali, Hack has been able to determine the cephalin, lecithin, and sphingomyelin contents of tissues. The determination of serine and ethanolamine in phospholipids has been described by Burmaster (59).

Progress has also been made in the preparation and characterization of pure phospholipids. Pure egg lecithin has been prepared by Sinclair (60), and pure sphingomyelin has been obtained from beef lung by Thannhauser *et al.* (61). The latter also identified the component fatty acids of the sphingomyelin. The preparation of dipalmityl lecithin from animal tissue has been reported for the first time by Thannhauser *et al.* (62). Macpherson & Lucas (63) isolated an inositol-containing phospholipid from rat liver which differs apparently from the inositol phospholipids of soybean (64) and of brain (65). The composition of cardiolipin has been reported by Pangborn (66).

LIPOTROPIC FACTORS

Choline.—Horning & Eckstein (67) have shown that the lipotropic effect of choline and of methionine in rats can be demonstrated as early as eight hours after a single oral administration. With the aid of radioactive phosphorus they studied phospholipid turnover in the liver and found that lipotropic action could take place without any apparent increase in the amount of radiophospholipid formed by the liver; sometimes increased amounts of phospholipid P^{32} were present after the feeding of choline or methionine without a corresponding fall in liver lipids. According to these authors, the effect of choline and methionine on phospholipid turnover may not be connected with their lipotropic activity.

The stimulating effect of choline on lipid phosphorylation has again been demonstrated by Artom & Cornatzer (68). In both liver and small intestines of rats fed a low-fat, low-protein diet, lipid phosphorylation remained practically unchanged following the administration of a single dose of fat by stomach tube. On the other hand, lipid phosphorylation in both tissues was increased by choline, and a still greater increase occurred when choline and fat were given simultaneously.

The effectiveness of choline in inhibiting the deposition of cholesterol esters and glycerides in the livers of rats fed diets rich in cholesterol has been reaffirmed by Ridout *et al.* (69). No impairment of the lipotropic activity of choline was observed even when the test was prolonged to four months.

The mechanism of the lipotropic action of choline has been reviewed by Borglin (70). He and Abdon compared the oxygen consumption of muscle pulp obtained from normal and choline-deficient rats (71). The oxygen uptake was much slower with preparations made from deficient animals. This defect in the muscle metabolism of young animals made its appearance quite early, much sooner than the time required for the development of fatty livers. These workers believe that choline acts by being part of a coenzyme or biocatalyst necessary in intermediary metabolism.

Proteins and amino acids.—An approach to the vexed question whether the lipotropic action of casein can be equated with its methionine content has been made by Rose, Machella & György (72). These workers produced fatty livers in rats by feeding them for twenty-one days a diet consisting of 8.5 parts of an amino acid mixture, 47.5 parts of cane sugar, 40 parts of crisco, 4 parts of a salt mixture, in addition to vitamin supplements. The amino

acid mixture was designed to contain the essential amino acids (with the exception of methionine) in the proportions in which they occur in casein; glutamic acid was used to make up the non-essential fraction. Since the daily administration of 50 mg. of methionine failed to reduce significantly the fat content of the liver, they suggest that the methionine content of casein *per se* does not account for the lipotropic effect of a high-casein diet. Various explanations of this finding are pointed out.

Pancreas.—Proof of the presence of an unidentified factor in pancreas that regulates fat metabolism rests on the observation that fatty livers develop in completely depancreatized dogs maintained with insulin and fed a high-protein diet, and on the additional fact that this fatty liver can be prevented by the ingestion of as little as 60 mg. per day of a choline-free fraction derived from raw pancreas. Since the addition of free methionine to a diet already containing adequate amounts of protein was found effective in preventing fatty livers, it was proposed as a working hypothesis that this pancreatic factor acts upon ingested proteins within the intestinal tract to release their lipotropic amino acids (73). Support for this hypothesis has now been provided (74). In contrast to unhydrolyzed protein which, even when fed in very large amounts, fails to stop the development of fatty livers, hydrolyzed casein readily prevented fatty livers. The antifatty liver action of 20 gm. of hydrolyzed casein in the completely depancreatized dog was accounted for by its methionine content.

An earlier observation, that orally administered pancreatic juice also prevents fatty livers in completely depancreatized dogs maintained with insulin, was confirmed by Montgomery *et al.* (75). These workers showed that the external secretion of the pancreas is highly active in this respect. The daily ingestion by a 10 kg. dog of as little as 10 cc. of pancreatic juice, a small fraction of the daily output of the pancreas, was found sufficient to keep the fat content of the liver normal.

Miscellaneous.—Ludewig & Chanutin (76) report that rats with renal insufficiencies produced by subtotal nephrectomy deposit less fat in the liver. This was shown for a large variety of diets, including some that were choline-free. By using large amounts of N¹-methylnicotinamide (2 to 4 per cent) Najjar & Ratcliffe were able to decrease the amount of liver fat in rats fed diets containing high fat or glycocyamine (77). Gillman & Gillman (78) found that the fat content of the livers of patients with infantile pellagra

decreased after ingestion of a dried stomach preparation. However Forbes & Petterson were unable to detect lipotropic activity when such a preparation was fed to rats maintained on a low-choline, low-protein, high-fat diet (79).

CHOLESTEROL METABOLISM

The adrenal cortex is extremely rich in cholesterol. According to Long (80) the adrenal cholesterol content of the adult white rat kept under resting conditions amounts to about 4 per cent. It is also of interest that approximately 90 per cent of this cholesterol is esterified, as compared with 50 per cent in the liver and 10 per cent in the brain. Studies on the conditions that alter the concentration of adrenal cholesterol have done much to elucidate its metabolic significance (80 to 83). A single injection of adrenotropic hormone, which is the only means by which an increased secretion of cortical hormone may be provoked, was shown to reduce considerably the cholesterol content of the gland in a few hours (80, 81, 83). The specificity of this response is revealed by the failure of the injected hormone to alter the cholesterol content of blood plasma, liver, spleen, brain, heart, skeletal muscle, or lymph nodes. The evidence marshalled by Long (80, 81) suggests that cholesterol is actually the precursor of adrenal steroid hormones. This view is supported by the recent work of Bloch (84), who with the aid of deuterium as a labeling agent demonstrated the conversion of cholesterol to pregnanediol.

The recent work of Claesson & Hillarp (85) is of interest in the above connection. These workers have identified by optical and histochemical procedures a cholesterol-like compound in the interstitial cells and the theca interna of the ovary. Because its amount fluctuates with the sexual phase of the animal, being high, for example, during estrus and pregnancy, and because its amount falls to very low levels after administration of gonadotropic hormone, they suggest that it is the precursor of the estrogenic substances formed in the rabbit ovary. The high concentration of the anisotropic estrogen precursor described by these investigators in the interstitial tissue of the ovary, the small amount in the theca interna, and the absence of this material from the follicular fluid indicate a different site of origin of estrogen from that usually postulated, or, perhaps, an alteration in the chemical and physical properties of this material during its transfer into the liquor folliculi.

The synthesis of cholesterol from acetate and acetate precursors has been reviewed by Bloch (86). Experiments with labeled acetate have shown that acetic acid contributes to the entire steroid molecule and that the higher fatty acids or intermediates of fatty acid metabolism do not lie in the path of acetate-cholesterol conversion.

The cholesterol content of the adrenal, skin, liver, and muscle of rats fed various diets has been studied by Abelin. The addition of unsaturated fat to the diet increased the cholesterol content of these tissues (87). Abelin also reported that the cholesterol content of various organs remains within the normal range in growing rats fed exclusively on proteins (88). He has also reviewed some phases of the metabolism of cholesterol (89).

Relation of cholesterol to tissue degeneration with particular reference to atheromatosis.—It is now clear that a sustained hypercholesterolemia injures arterial tissue. The production of atheromatosis by cholesterol feeding has been well established for the rabbit, bird, and guinea pig. The age of the rabbit, according to Pollak (89), is of importance in determining whether cholesterol feeding will produce atherosclerotic lesions. Pollak produced them readily in rabbits one year of age but failed to observe them in five, ten and twenty-five-week-old rabbits, even though the hypercholesterolemia present in all age groups was of equal degree.

The opinion has been expressed that the production of atheromatous aortic lesions by cholesterol feeding is limited to a few species. Thus Hueper (90) failed to find them in *Macacus Rhesus* monkeys fed cholesterol. They can, however, be induced in the dog provided sufficiently high blood cholesterol levels are maintained. Steiner & Kendall (91) observed gross lesions in dogs after the serum cholesterol had been maintained at levels from three to ten times the normal for at least twenty-seven weeks. This was accomplished by feeding each dog daily 10 gm. of cholesterol along with thiouracil, which by depressing thyroid function assisted in raising the blood cholesterol level.

The distinctive features of the lipid metabolism of the bird have made possible the development of a new method for the study of experimental atherosclerosis. Lindsay *et al.* (92) induced atheromatous changes in the artery of this animal by the subcutaneous implantation of diethylstilbestrol, a procedure that results in a sustained hyperlipemia. They point out that the atherosclerosis so produced more closely resembles spontaneous lesions in birds than

does that produced by the exogenous administration of cholesterol.

An increase in the cholesterol content of the atherosclerotic artery has repeatedly been demonstrated. Indeed, the lipid concentration in the intimal lesions rises as the atherosclerotic process becomes more severe (93). Moreover, even in the absence of retrogressive change, the lipid content of the media increases with advancing age. A progressive increase with age in the cholesterol content of the aorta of normal persons was recently confirmed by Faber (94). But the increased lipid content of the artery that occurs with age in normal men is neither preceded nor accompanied by a rise in the level of plasma cholesterol. The most satisfactory evidence on this point has been provided by Landé & Sperry (95), who compared the degree of atherosclerosis of the aorta with the concentration of serum cholesterol in 123 healthy persons who died suddenly from violence. They concluded that the incidence and severity of arteriosclerosis are not directly affected by the level of cholesterol in the blood serum. Such observations have led Moreton (96) to postulate that the physical state and particle size of the plasma lipids determine whether lipids are deposited in the artery. During the three to five hours following a fat meal, according to this investigator, there appear in the blood stream greater numbers of lipid particles of considerably larger size than those found in normal fasting plasma or after a fat-free meal. He believes that transient showers of large lipid particles in plasma induced by fat meals over a life time may be the cause of the lipid deposition found in human atherosclerosis.

It is not surprising that the above considerations have led to much recent discussion on the role of dietary cholesterol (egg yolk) in promoting arteriosclerosis in normal man (97, 98). Since it has been demonstrated that acetic acid is a precursor of cholesterol (86), the rate of cholesterol synthesis by the liver may be of more importance than ingested cholesterol in determining whether cholesterol is deposited on the artery.

Kennedy & Okey (99) have confirmed their earlier observation that when guinea pigs are fed a diet containing one per cent cholesterol in 12.5 per cent fat for about fifty days they develop, in addition to fatty livers, a severe anemia, an enlarged spleen, hyperplastic bone marrow, iron deposits in the proximal convoluted tubules of the kidneys, and thickened alveolar lung walls containing macrophages laden with iron and fat. They have now shown that splenectomy does not prevent the development of these

tissue changes. The appearance of a macrocytic anemia in cholesterol-fed rabbits has also been reported by Dubach & Hill (100). According to Altschul, cholesterol feeding in rabbits and guinea pigs stimulates heterotopic blood formation, particularly in the adrenal cortex (101).

LIPID METABOLISM OF THE FETUS

Goldwater & Stetten (102) applied the isotope tracer technique to a study of the passage of lipids across rat placenta. Pregnant rats were fed deuterio fatty acids for two days between the eighteenth and twentieth days of gestation and then were sacrificed. The deuterium concentration of the fatty acids isolated from the fetus provided further evidence for the view that intact fatty acids can cross the placenta from mother to fetus. These workers also fed deuterio-cholesterol to pregnant rats for seven days. Since the concentration of D_2O recovered in fetal body water was too low to account for the concentration of deuterium in the cholesterol of the fetus, they concluded that ingested cholesterol can cross the placenta.

When D_2O was fed to pregnant rats, the deuterium content of the fetal fatty acids and cholesterol soon exceeded that of the maternal liver. This indicates that the fetal rat approaching term is capable of independently synthesizing both fatty acids and cholesterol. Goldwater & Stetten estimate that at about the twentieth day of its development the rat fetus synthesizes half of its fatty acids in about 1.5 days and half of its cholesterol in about 2.5 days.

According to Popják (103) the concentration of lipids in the plasma of the fetal rabbit is much higher than that in the maternal plasma and is not readily influenced by the presence of a lipemia in the mother. This is not the case in man (104) and sheep (105). Sadowsky *et al.* (104) report that at birth the cholesterol level in the fetal blood is lower than in the maternal blood but that the latter is able to influence the cholesterol level in the former. Popják (103) fed pregnant rabbits a high-cholesterol diet. Storage of cholesterol was observed in maternal liver and placenta but not in the fetus.

INTERMEDIARY METABOLISM OF LIPIDS

In confirmation of earlier work by Weinhouse *et al.* (106) Buchanan, Sakami & Gurin (107) found that carboxyl-labeled

octanoic acid gave rise to acetoacetate in which both the carboxyl and the carbonyl carbons were labeled. In the experiments of the latter investigators, however, the carboxyl carbon contained a higher concentration of C^{13} (0.94 atom per cent excess C^{13}) than did the carbonyl carbon (0.62 atom per cent excess). Their work, as well as that of Weinhouse *et al.*, demonstrates that the carboxyl carbon of octanoic acid is an important precursor of both the carboxyl and the carbonyl carbons of acetoacetate.

In order to test whether, in the above experiment, the appearance of C^{13} in the carbonyl carbon of acetoacetate might be explained by the theory that the carboxyl-labeled octanoate splits into two acetoacetate molecules and the acetoacetate formed is then rapidly broken down to two-carbon fragments which recondense at random, Buchanan *et al.* (107) incubated two types of isotopic acetoacetate (carbonyl-labeled and carboxyl-labeled) with liver slices. The concentrations of C^{13} found in the carboxyl and carbonyl carbons at the end of the incubation period led them to conclude that acetoacetate is not split into two-carbon fragments which recondense at random into acetoacetate again. They favor the view that fatty acids are oxidized by the removal of two-carbon fragments, which then condense to acetoacetate only when conditions favorable for the complete oxidation of the fragments do not prevail. According to this view, acetoacetate is not in the main path of oxidation of fatty acids.

In an article entitled the "Metabolism of Acetic Acid in Animal Tissues," Bloch (108) has dealt with the present state of our knowledge of the mechanism of breakdown and synthesis of fatty acids. He, too, concludes that it is unnecessary to assume that acetoacetate lies in the main path of fatty acid oxidation. Successive beta oxidation followed by removal of two-carbon fragments appears to be the principal event in fatty acid oxidation. Synthesis of fatty acids, according to Bloch, probably occurs through the condensation of two-carbon units with acetate and higher fatty acids and the subsequent reduction of the keto compounds to saturated fatty acids. Thus the mechanism of fatty acid synthesis is brought about by the reversal of the steps in their degradation.

Bloch deals critically with the view that the two-carbon fragment arising from fatty acid oxidation is identical with that arising from carbohydrate oxidation. He points, for example, to the contrasting behavior of labeled fatty acids and pyruvate in the acetylation of foreign amines in the intact animal.

Additional evidence that the tricarboxylic acid cycle provides a pathway for the oxidation of fatty acids has been obtained by Weinhouse, Medes & Floyd (109). These workers studied the aerobic oxidation of carboxyl-labeled and carbonyl-labeled acetoacetate by rat kidney homogenates to which oxaloacetate had been added. They were able to isolate citric acid containing a high concentration of C^{13} in the primary carboxyl carbons. This would appear to provide indisputable evidence for the participation of citric acid in the oxidation of the acetoacetate. Weinhouse *et al.* believe that the component which actually condenses with the oxaloacetate is a two-carbon fragment arising from the breakdown of acetoacetate, and not acetoacetate itself as postulated by earlier workers.

The possibility that acetate itself is an intermediate in the oxidation of acetoacetate, via the tricarboxylic acid cycle, by kidney extracts (cell-free) has been considered by Buchanan *et al.* (110). It was found that acetoacetate is utilized much more rapidly than acetate by such extracts. Furthermore, when isotopic acetoacetate was incubated in the presence of nonisotopic acetate and α -ketoglutarate, the latter contained a much higher concentration of C^{13} than the acetate at the end of the incubation period. They therefore concluded that acetate itself is not a direct intermediate in acetoacetate oxidation. However, the possibility that some acetate is formed during the metabolism of acetoacetate was not completely ruled out.

The rate and site of acetate metabolism in the body have been studied by Smyth (111). He has shown that in the intact animal the liver accounts for about half the acetate used, a much greater fraction than is suggested by experiments *in vitro*. The kidney, on the other hand, is not as prominent in acetate metabolism of the intact animal as is indicated by observations *in vitro*. The participation of muscle in acetate metabolism is discussed.

Breusch & Ulusoy (112) have studied the metabolism of β,δ -diketohexanoic acid in the presence of various tissue suspensions. The finding that the compound was rapidly oxidized by a liver suspension with the formation of 1.3 molecules of acetoacetic acid is taken as evidence for the Hurler-Jowett-Quastel theory of alternate β -oxidation in the liver. However, other organs such as kidney, muscle, brain, and lung do not metabolize β,δ -diketohexanoic acid to a measurable extent. It appears that in these tissues fatty acids are not degraded by alternate β -oxidation, but

rather by a simple β -oxidation (Knoop) followed by breakdown through the tricarboxylic acid cycle.

Breusch & Tulus (113) reported that the capacity of minced tissue immediately after death to metabolize γ -keto-valeric (levulinic) or δ -ketocaproic acids was negligible, although α - and β -keto acids were readily utilized.

BLOOD LIPIDS

A critical review of the value of serum lipids in the diagnosis of disease in man has been made by Thannhauser (114). The lipid disturbance underlying various diseases, particularly xanthomatous diseases, is briefly considered. Laboratory procedures which he considers most reliable for the determination of phospholipids, cerebroside, cholesterol and its esters, total fatty acids, and neutral fat are mentioned. A table containing the analysis of serum lipids in various diseases is presented.

Bodansky (115) found that the ratio of free to total cholesterol in normal serum is much more constant than is usually reported. The normal percentage ranged from 27 to 30. Consistent successive values above 30 per cent were taken to indicate organic or functional liver disease. In confirmation of earlier work, Foldes & Murphy (116, 117) have shown that the content of total cholesterol, ester cholesterol, and phospholipids of plasma and cells of a young group (twenty to thirty-five years of age) of healthy subjects did not differ from the values found in an old group (seventy to ninety years of age). These workers have also studied the levels of these three lipid constituents in the plasma and cells of patients suffering from thyroid disease.

A gradual decrease in the total cholesterol content of plasma of calves from birth to twenty-three weeks of age is reported by Teeri, Keener & Morrow (118).

REVIEWS

In addition to those already referred to, the following reviews are worthy of note. Peters' chapter on lipids covering more than two hundred pages in the text *Quantitative Clinical Chemistry* is a comprehensive treatment of lipid metabolism in both normal and pathological states (119). Its bibliography of over nine hundred papers is a ready reference source for investigators in this field. Lipid metabolism has also been reviewed by White (120). A

compilation of the recent literature on fats, oils, and soaps has been prepared by Piskur (121); a section on biochemistry and physiology has been included. In an article entitled the "Interrelation of Carbohydrate and Fat Metabolism," Deuel & Morehouse have dealt with the transformation of carbohydrate to fat, the conversion of fat to carbohydrate, and the relation of ketosis to carbohydrate oxidation (122). Hevesy (123) has prepared an extensive review of the experiments on phospholipid metabolism in which radioactive phosphorus was used as an indicator.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS¹

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A review of protein metabolism is the story of amino acids, singly and in various combinations, as they enter into, and become a part of, the chemical matrix of living systems. Whipple and others associated with the Rochester group (1) have visualized body proteins in dynamic equilibrium within this matrix so that transfer of amino acids, or combinations of them from one tissue to another, is a continuous and normal process. Thus, body protein stores, together with protein anabolism and catabolism, form a nicely balanced steady state, the reality of which is demonstrated by studies involving isotopes (2).

THE RETENTION OF DIETARY NITROGEN

Maintenance of nitrogen equilibrium.—The establishment of nitrogen balance (NB) in the adult animal is an expression of this steady state, an over-all description of which is written in the following equation:

$$NB = K(AN) - NE_0 \quad (I)$$

where AN is absorbed food nitrogen, and NE_0 is the excretion of nitrogen on a protein-free diet (3, 4). This is an equation for a straight line in the region of negative nitrogen balance and K is the slope of that line. Expressed in terms of urinary nitrogen, UN, the steady state becomes:

$$UN = (1 - K)AN + UN_0 \quad (II)$$

where UN_0 is the excretion of urinary nitrogen on a protein-free diet. Values for NE_0 and UN_0 represent, therefore, minimum catabolism of nitrogen which will permit the establishment of nitrogen equilibrium (3). It can be argued that this minimum excretion of nitrogen represents the catabolic endogenous nitrogen proposed by Folin, the reality of which is emphasized by Block & Mitchell (5). The dynamic equilibrium between tissues and surrounding

¹ This review covers the period from September, 1946 to September, 1947.

media does not permit, however, an easy differentiation of food nitrogen from body nitrogen (6).

Values for NE_0 or UN_0 vary according to the physiological state of the animal, reflecting to a large extent the magnitude of the protein stores (7, 8). If NE_0 represents the excretion of body nitrogen at all nitrogen intakes, then K is the fraction of absorbed nitrogen retained in the body of the animal. This fraction has been called the "biological value" of the dietary protein, a value that never can be greater than unity. Values for K greater than unity have been obtained, however, demonstrating that excretion of body nitrogen can decrease with nitrogen intake, absorbed nitrogen sparing body nitrogen (6, 9, 10, 11). K , therefore, is some function of, but not necessarily equal to, the "biological value" of the protein source. Since K is the rate of change of nitrogen balance with respect to nitrogen intake, Allison, Anderson & Seeley (6) have called it the nitrogen balance index of the dietary protein. This index is the tangent to the curve relating nitrogen balance to nitrogen intake and it is constant in the region of negative balance, but becomes a variable in the region of positive nitrogen balance. The effects of the excretion of minimum catabolic nitrogen, UN_0 , upon the nitrogen balance indexes of proteins fed orally, or hydrolyzates fed intravenously, to dogs have been determined by Allison, Seeley & Ferguson (12). They found that the nitrogen balance index for any one protein was constant under standardized conditions if UN_0 was greater than 60 mg./day/kg. of body weight, but that the index increased as UN_0 decreased below this value in dogs depleted in proteins.

The feeding of some single amino acids will even depress the excretion of body nitrogen in animals receiving a protein-free or low nitrogen diet. This reduction is most marked when methionine is fed to dogs or rats (11, 13). Brush *et al.* (13) have found also that cystine, choline, and all of the essential amino acids, except phenylalanine, valine, and tryptophane, exert some action in sparing body nitrogen in the rat. The depression of so-called endogenous metabolism by the addition of dietary nitrogen has caused these authors to question the reality of a distinction between endogenous and exogenous metabolism. For that reason, Swanson and associates (10) have used the term, "biological efficiency" instead of "biological value" to describe their data, the value for efficiency having the same significance as nitrogen balance index in the region

of negative nitrogen balance. Another new method of bioassay involving maintenance of a standardized physiological state has been developed by Tomarelli & Bernhart (14), a method which measures the amount of nitrogen necessary to maintain constant weight in protein depleted rats.

Maintenance and growth.—Originally the concept of "biological value" referred to the fraction of nitrogen retained in the adult animal for maintenance. Mitchell and co-workers (15, 16, 17) extended the measurement of "biological value" to include the sum of the fractions of nitrogen retained in the animal for growth and for maintenance. Goyco & Asenjo (18) have applied these techniques recently to the determination of the "net protein" values of food in young rats. The relative participation of dietary nitrogen in growth and maintenance in the rat, however, has been shown by Barnes, Bates & Maack (19) to vary markedly depending upon the amount and the nutritive quality of the protein. Their results demonstrated that the percentage of protein utilized for growth passes through a maximum as the amount of absorbed protein increases, while that utilized for maintenance decreases. The sum of the fractions utilized for growth and maintenance is the "biological value" of the protein, a value which decreases as absorbed nitrogen increases, being highest at low levels of intake when the animal is in negative or low positive nitrogen balance. A decreasing "biological value," with increasing protein intake in growing rats, has its counterpart in the adult animal. Allison & Anderson (3) have shown that equation I, for example, becomes curvilinear in the region of positive nitrogen balance, the nitrogen balance index decreasing in this region as absorbed nitrogen, AN, increases. Barnes and associates (19) pointed out, that since the protein requirements for growth and maintenance are different, these two factors should be measured independently.

The methods involving growth which are used most extensively for determining the nutritive quality of proteins are derived from the studies of Osborne, Mendel & Ferry (20). Recent applications of this method are found in the following references (21 to 28). In most of these studies the nutritive quality of a protein is expressed as the ratio of body weight gain to protein consumed, the protein efficiency ratio. Osborne and co-workers (20) stipulated that the maximum ratio should be established by feeding varying amounts of protein, and that this maximum be taken as the index of the

value of the protein for growth. Few laboratories have followed this stipulation, however, feeding instead a constant amount of protein (usually 10 per cent) in the diet. A study of the relation between protein efficiency and gain in weight, on diets of constant protein content, has been made by Hegsted & Worcester (29). They found a very high correlation between gain in weight and protein efficiency, the latter variable being a function of the former rather than characteristic of the protein fed. Barnes & Bosshardt (30) have re-examined the protein efficiency method in rats and applied it also to mice. They emphasized that the common practice of feeding a constant amount of protein can result in considerable distortion of nutritive values. Harte, Travers & Sarich (31) found that the variance in growth response in rats was only about one-eighth to one-tenth of that observed for animals fed *ad libitum* if the food intake was partially restricted. There is great need, therefore, to standardize the growth procedure so that results from different laboratories may be compared. Barnes, Bosshardt and associates have been developing such standardized procedures, applying them particularly to the mouse (30, 32). They have demonstrated that the utilization of ingested, or preferably absorbed, protein for body protein gain is the most valid expression of protein utilization for growth. For the calculation of absorbed protein Bosshardt & Barnes (33) developed a method for the determination of metabolic fecal nitrogen and protein digestibility in the mouse. Their data indicate that metabolic fecal nitrogen may be different on protein-free or low protein diets, than under conditions of protein feeding. Using metabolic fecal nitrogen measurements to calculate absorbed protein, they determined the per cent utilized for body gain and the true dietary levels for maximal utilization.

Dunn & Rockland (34) have introduced a new and unique method for the determination of "biological value" of proteins by use of the ciliated protozoan, *Tetrahymena geleii* H. They are comparing the results obtained with nutritive values measured in higher animals.

Calories and nitrogen retention.—Some effects of caloric intake on nitrogen retention have been studied by Allison and associates (3, 6) who have reported that UN_0 increases when the caloric intake of dogs is reduced below the optimum. Similarly, Johnson and co-workers (35) found that a minimal excretion of nitrogen, UN_0 , could not be established in man on reduced caloric intakes. The

nitrogen balance index was not altered, however, in the dog, until the caloric intake was over 50 per cent below the optimum, the index being reduced markedly when the caloric intake was lowered to 25 per cent of that which was considered adequate. Willman *et al.* (36) also found a reduction in "biological efficiency" in the rat and Schwimmer and associates (37) demonstrated a reduction in the utilization of protein in man when the caloric intake was reduced markedly. Bosshardt *et al.* (38) have obtained data on rats and mice which correspond to the first type of response where the changes in caloric intakes are small. They found that there was a range of caloric intakes that maintained maximal protein utilization, but decreases below that range resulted in a reduction in protein efficiency ratios. Thus reduced caloric intake may increase "endogenous" excretion of nitrogen and leave a smaller proportion of the absorbed protein for formation of new tissues.

A preliminary report has been made by Bosshardt and co-workers (39) on the influence of fat and carbohydrate calories on protein utilization. They found that on a restricted caloric intake (approximately 75 per cent of the minimum required for optimal protein utilization) the nitrogen of wheat gluten was utilized more efficiently by the mouse than the nitrogen of casein. No difference was noted in the protein sparing action of fat and carbohydrate calories. Similarly, Allison, Anderson & Seeley (6) found fat and carbohydrate calories to have equivalent protein sparing action in the dog. Swanson *et al.* (10, 36), on the other hand, demonstrated in rats a marked protein sparing action of fat, more so than carbohydrate, when the caloric intake was reduced. Scheer and co-workers (40, 41) have shown that during a period of severely restricted but isocaloric feedings, weanling rats grew better on diets containing fat than on similar diets lacking in fat. Indeed, Deuel *et al.* (42) found that optimum growth occurred on diets containing 20 to 40 per cent fat, a growth that resulted in greater physical ability and better reproduction and lactation. Restriction of caloric intake in rats has been shown by Miller, Friedman & Deuel (43) to reduce values for hemoglobin, plasma proteins and hematocrits. When the caloric restriction is most severe, as in fasting, a ketosis develops which is a function of the protein and carbohydrate stores in the animal. Tidwell & Treadwell (44) suggested that the greater fasting ketosis observed after a low protein intake may be correlated with an increased utilization of carbohydrate.

UTILIZATION OF AMINO ACIDS

The essential amino acids.—Modern research on the nutritive role of amino acids began when mixtures of purified amino acids replaced proteins in the diets of animals, researches which were inaugurated by Rose in 1930. From these studies it was discovered that a mixture of ten of the amino acids, in suitable proportions, is an efficient source of nitrogen for many complex physiological functions in the rat. Recently, Rose (45) has extended these studies to man by feeding a protein-free diet plus an amino acid mixture to healthy male graduate students. The amino acids used in the first experiments were those found essential to animals. The results demonstrated that valine, methionine, threonine, leucine, isoleucine, phenylalanine, tryptophane, and lysine are necessary constituents in the diet of man, but that histidine and arginine are not necessary for the maintenance of nitrogen equilibrium in adults. Rose pointed out, however, that under the stress of growth, or disease, or special functions, histidine or arginine may become essential to man. Quantitative studies have been and are being conducted to determine the minimum level for each amino acid, compatible with consistent nitrogen balance. In a review of the amino acid requirements of man, Albanese (46) has emphasized also that the essentiality of an amino acid may not always be indicated by nitrogen balance studies. Harte & Travers (47) calculated human amino acid requirements for nitrogen balance. They pointed out that nutritive values of proteins may be determined largely by the essential amino acid content, and that the fraction of absorbed nitrogen excreted in the urine may reflect the excess nonessential amino acids which cannot be utilized for lack of an optimum quantity of essentials. Geiger (48) has given additional evidence that "incomplete" amino acid mixtures are not stored in the body.

Rose & Womack (49) have expanded their studies in animals to determine the relative availability of enantiomorphs of the essential amino acids and proved that D- and L-phenylalanine have practically equal nutritive values for the growing rat. Womack & Rose (50) demonstrated also that approximately half of the phenylalanine may be replaced by tyrosine. Luckey *et al.* (51) obtained data proving that chicks grow subnormally on a synthetic diet in which amino acids are the sole source of nitrogen. Casein proved superior to its component amino acids in supporting growth in the

chick, and growth on the eleven amino acids essential to the chick was improved by adding nonessentials. Grau & Peterson (52) showed growth to be best in the chick when the diet contained 0.5 per cent L-isoleucine, 1.5 per cent L-leucine, and 0.7 per cent L-valine. The isomers, D-isoleucine and D-valine, were not utilized for growth, but DL-leucine was as effective as L-leucine. Grau (53) and Kratzer *et al.* (54) have studied the essential amino acid deficiencies in food proteins in the chick. Kratzer and co-workers (55) have reported on the arginine requirement of young turkey poults. Grau, Kratzer & Asmundson (56) obtained results indicating that the L-lysine requirement is higher for the poult than for the chick. The high protein requirements of the poult have been investigated by Fritz *et al.* (57).

Nutritive value and essential amino acids.—Rose and co-workers (58) have pointed out that an essential amino acid is defined, at the present time, as one which cannot be synthesized from materials in the diet at a speed commensurate with demands for normal growth. They recognize, however, that this definition may not cover other functions such as reproduction and detoxication, or even maintenance. Lysine, for example, is an essential amino acid for growth in the young rat, but Mitchell (59) has demonstrated that this acid is not essential for the maintenance of nitrogen equilibrium in the normal sexually mature adult rat. On the other hand, it is required for maintenance of nitrogen equilibrium in the dog (60) and in protein nutrition in adult man (45). Mitchell suggests that in a rapidly growing animal, such as the rat, synthetic reactions leading to the formation of lysine may be inadequate for growth but adequate for maintenance. This is only true, however, when there is no growth or regeneration of tissue proteins in the adult (61). Although arginine can be excluded from the diets of rats without loss in weight, Borman *et al.* (58) have demonstrated that it is a necessary dietary component for optimum growth, the synthesis *in vivo* not keeping pace with the maximum arginine requirements. These authors found also that arginic acid is not equivalent to arginine in growth promoting properties, leading to the conclusion that the hydroxy compound is not readily transformed to the amino acid by the rat. The work of Gingras *et al.* (62) showed, too, that DL- α -amino- ϵ -hydroxycaproic acid could not replace lysine as an essential amino acid in the rat.

The species differences in the quantitative requirements of an

amino acid are emphasized by the work of Cox and associates (63) who found that the addition of cystine or methionine to a casein hydrolysate increased the rate of growth in rats and that the addition of methionine increased nitrogen retention when the hydrolysate was fed intravenously to dogs, but that the addition of methionine did not increase nitrogen retention in man. Cox *et al.* (63, 64) found, too, that casein and lactalbumin were equivalent in the maintenance of nitrogen equilibrium in man, while lactalbumin was superior to casein in the rat and the dog. In this connection, it is interesting to note that the superiority of lactalbumin over casein in promoting growth in rats could not be demonstrated by Daniel & Harvey (65) if the lactalbumin was not dialyzed free of inorganic constituents found in whey. Hegsted *et al.* (66) found species differences when comparing the nutritive values of the proteins in mixed diets of dogs, rats and human beings.

Murlin and associates (67) have published a series of papers on the "biological value" of proteins in relation to the essential amino acids which they contain—studies on the maintenance of nitrogen equilibrium in adult man. They investigated the excretion of so-called endogenous nitrogen in man (UN_0 in equation II), finding variations in UN_0 similar to those which have been described in experimental animals. A steady state was established, however, so that relatively constant values for UN_0 could be measured, values which were correlated with body weight. Thus in their second paper Murlin *et al.* (68) determined the "absolute biological value" of egg and soybean proteins. They demonstrated an interconvertibility of "biological values" from one "endogenous" level to another, which proved the validity of equations I and II as applied to experiments in man. The authors studied, further, the effect of adding single essential amino acids to egg or soybean protein. They found that such an addition reduced the "biological value" of these proteins. In the third paper (69) they pointed out that essential amino acids, compounded in the proportions occurring in various proteins, did not possess as high a "biological value" as that of the protein. Low "biological values" of mixtures of amino acids were shown to be the result of the presence of unnatural isomers. The fourth paper (70) in the series is a record of analyses of fifteen protein foods for the ten essential amino acids. Further proof that the unnatural isomers are not utilized well is furnished in the fifth paper (71). Data are presented also which show that

individual essential amino acids greatly differ in retention potency.

Block & Mitchell (5) have written a comprehensive review on the correlation of the amino acid composition of proteins with their nutritive value, recording analyses of fifty-eight proteins, or protein mixtures. These correlations were found to be of great value in explaining, and in predicting, the biological utilization of proteins. Chick (72) has correlated also the nitrogen balance and growth methods with the amino acid contents of proteins. Russell *et al.* (25) found that the availability of methionine in legumes varied with the variety, and that the nutritive value in the rat could not be predicted from an analysis for this amino acid, emphasizing the error that may be encountered in using the results of the amino acid analyses of proteins for predicting nutritive values. Lack of good correlation between amino acid analyses and nutritive value is due, often, to the effects of food processing and digestion (73).

Effects of food processing and digestion.—It is known, for example, that proper heat treatment will increase the nutritive value of soybean protein. Evans and associates (74, 75, 76) and Clandinin *et al.* (77) have presented data to show that moderate heating of raw soybean meal increased the nutritive value for the chick, while overheating decreased this value, and that moderate heating improved the digestibility of the meal by the chick or by trypsin and erepsin *in vitro*. Riesen and co-workers (78) demonstrated that prolonged heating resulted in a decreased liberation of lysine, arginine, and tryptophane by acid hydrolysis. Proper heat treatment increased, while excessive heating decreased, the liberation of essential amino acids by pancreatic hydrolysis. Melnick, Oser & Weiss (79) have suggested that

for optimum utilization of food proteins all essential amino acids must not only be available for absorption, but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation.

Their data on soybean protein indicated, for example, that methionine is liberated more slowly during digestion than leucine or lysine and that heat treatment of this protein increases the rate of liberation of methionine.

The effects of amino acid structure on absorption from the gastrointestinal tract are illustrated by the work of Schofield & Lewis (80). They found that the amino group in the alpha position favored absorptive processes, that the replacement of hydrogen by an hydroxyl group reduced the rate of absorption, and that the

D-isomer of alanine was less rapidly absorbed from the gut than the stereoisomer, L-alanine.

The effect of processing of food stuffs on the availability of lysine has also been studied recently (81). Block *et al.* (82) prepared a food in which proteins were mutually supplementary with a distribution of essential amino acids approximating that of whole egg. The raw cake had a very high protein efficiency (P.E. 3.3 to 3.5). Baking and drying, or toasting of the cake, reduced the protein efficiency to values as low as 0.7. Addition of lysine restored the nutritive value (P.E. 3.2). The authors suggested that heat treatment reduced the liberation of lysine by enzymatic digestion but not by acid hydrolysis. This suggestion agrees with the findings of Eldred & Rodney (83) who reported that heating casein reduced the enzymatic release of free lysine.

The effect of subdivision of the proteins on digestion and, therefore, on nutritive value, is illustrated by studies on keratins. Newell & Elvehjem (84) demonstrated that, in general, the rates of growth of chicks and rats fed rations containing keratins are correlated with the degree of subdivision of the proteins, good growths being obtained with lower levels of powdered hoofs if the rations were supplemented with lysine, tryptophane, and histidine.

The sulfur amino acids.—Diets low in casein (9 per cent) are used often to produce a methionine deficiency in rats. Li & Freeman (85), for example, used the 9 per cent casein diet to study the effect of methionine on protein-deficient rats exposed to benzene. Recently, Hall & Sydenstricker (86) found that a highly purified 9 per cent casein diet containing sucrose did not give normal growth with added methionine. They calculated this diet to be suboptimal in histidine, lysine, valine, threonine, and tryptophane. When these amino acids were added normal growth took place. When the sucrose in the diet was replaced by starch and inositol, and *p*-aminobenzoic acid and nicotinic acid were added, the 9 per cent casein plus methionine supported normal growth. The authors suggested that the deficiencies in this casein diet, other than methionine, are made up through intestinal synthesis when starch and the proper vitamins are added. The studies of Salmon (87) emphasize the deficiencies of a low casein diet, such as those used to produce cystine or methionine deficiency in rats. He observed that diets containing 18 per cent or less of casein were

deficient in labile-methyl groups, a deficiency that was made up by adding choline or methionine, but was aggravated by the addition of cystine or cystine and fat. Fat (30 per cent) in the diet tended to counteract the deficiency of nicotinic acid in these diets, indicating that a shift in energy metabolism from carbohydrate to fat spares nicotinic acid, this vitamin functioning primarily in carbohydrate metabolism. Sulfur amino acid deficiencies could not be demonstrated in low casein diets until labile-methyl and nicotinic acid deficiencies were remedied.

When the 9 per cent casein diet, supplemented with histidine, lysine, valine, and tryptophane, is fed to rats, a corneal vascularization is produced which is associated with a methionine deficiency. These corneal changes reported by Berg *et al.* (88) are similar to those found in rats fed a diet lacking in protein (89), except that the corneal vessels were usually much smaller and less numerous in the methionine-deficient than in the protein-deficient rats. Corneal vascularization is a tissue change associated with the absence of a number of different amino acids (90), the maintenance of normal corneal tissue, like other tissues of the body, requiring a certain complement of the essential amino acids (91).

A methionine-deficient diet can also cause hypoproteinemia due to a limited synthesis of cystine, an amino acid essential for the formation of plasma proteins. Data presented by Albanese and co-workers (92) show that hypoproteinemia and anemia are induced in rats on a methionine- and cystine-deficient diet. The deficiency in plasma protein, however, was not produced on a cystine-deficient diet in the presence of methionine. Matet, Matet & Fridenson (93) reported that liver necrosis, produced in rats by feeding a methionine-deficient diet containing uncooked soybean, was prevented or cured by the addition of cystine or methionine. The relationship between choline and methionine is illustrated by the work of Herrmann (94) who showed that this amino acid, like choline, acts as a decholesterolizing agent in old hens.

An optimum amount of methionine increased the nitrogen balance index of casein in the dog and the rat, conserving both body nitrogen and sulfur, while an excess decreased this index much below values obtained with casein alone. Excess methionine, in the rat, caused the destruction of body tissue nitrogen, but at the same time increased the production of liver protein and plasma globulin. Thus this abnormal pattern of amino acids caused the breakdown

of some tissue proteins and the building up of others [Allison, Anderson & Seeley (11); Brown & Allison (95)]. The work of Cox *et al.* (63) on the utilization of methionine agrees with the results of Johnson and co-workers (35) who have reported that the addition of DL-methionine to low protein diets of man does not decrease the excretion of urinary nitrogen. A comparison of data on man, with those obtained on the dog (11) and on the rat (13), lead them to conclude that methionine requirements are lower in man than in these animals. The experiments on man suggest that the addition of methionine provides an excess not needed in intermediary metabolism. Similarly, Schwimmer, McGavack & Drekter (37) have found methionine ineffective in promoting nitrogen retention in man. Tarver & Schmidt (96), using labeled methionine, demonstrated that this amino acid, given in small doses, was mostly retained in the body of the dog. Allison *et al.* (11) found that a definite quantity of methionine was required to produce the maximum sparing of nitrogen, a quantity that was completely retained in the animal. The sparing action was reflected in a reduction of the excretion of urea, and in the ratio between urinary ammonia and urea nitrogen. Excess methionine, over that needed to maintain sulfur balance, was metabolized and appeared largely as inorganic sulfate in the urine (97), a finding which is in agreement with studies made on cats, rabbits, and rats (98).

Amino acids in body fluids and tissues.—Recent advances in methods for the determination of amino acids have stimulated studies on the amino acid composition of body fluids and tissues. Prescott & Waelsch (99), for example, have determined the free and combined glutamic acid in human blood plasma. Gutman & Alexander (100) studied glycine and alanine and their relationship to the total amino acids in the blood of normal human subjects. Hoberman (101) determined guanidoacetic acid and arginine in human urine and serum. Bonsnes & Brew (102) reported on the plasma amino acid concentration during normal pregnancy, labor, and early puerperium. Harris & Harris (103) recorded data on the effect of insulin hypoglycemia and glucose on amino acids in the blood of mental patients. Frankl & Dunn (104) measured micro-biologically the free tryptophane, histidine, and cystine in normal human urine. Similarly, Harvey & Horwitt (105) determined the content of essential amino acids in human and dog plasma and Hier, Cornbleet & Bergeim (106) the amino acids of human sweat.

Schweigert and co-workers (107) determined the free tryptophane in blood and urine and demonstrated, among other things, that the free tryptophane content was a function of the amount in the diet. Steele and associates (108) correlated the amino acids in the urine of human subjects with diet. Sauberlich & Baumann (109) and Pearce, Sauberlich & Baumann (110) studied the effect of dietary protein upon amino acid excretion by rats and mice, demonstrating a much higher percentage of urinary amino acids in animals fed incomplete than those fed complete proteins. Grau (111) determined the phenylalanine and tyrosine content of chicks and eggs. Melampy (112) reported on some of the amino acids of chicken erythrocytes.

INTERMEDIARY METABOLISM

Shen & Lewis (98) have continued studies on sulfur metabolism and have shown

that oxidative deamination of the sulfur-containing amino acids, or their derivatives, may occur even though further oxidation (i.e., of sulfur to sulfate) is blocked.

The oxidation of the labile methyl group of methionine has been demonstrated in du Vigneaud's laboratories (113) by feeding this amino acid containing C^{14} to rats. The methyl carbon was most highly concentrated in the kidneys, liver, and adrenals. Dent (114) presented evidence to show that methionine can be metabolized to α -aminobutyric acid, accounting for the presence of this amino acid in the body. In studies involving isocysteine, Wingo & Lewis (115) have shown that administration of this amino acid to rabbits does not increase the excretion of oxidized sulfur in the urine, but does increase the organic sulfur fraction, mainly disulfide sulfur. The authors suggest that isocysteine, like β -alanine, resists deamination, and that it is possible that oxidation of sulfur in isocysteine, as in cysteine, must be accompanied or preceded by, oxidative deamination. Tarver & Schmidt (96), in their studies on urinary sulfur partition in normal and cystinuric dogs, demonstrated that radioactivity of cystine sulfur is higher in cystinuric than in normal animals. A close metabolic relationship was indicated between the components of the urinary neutral sulfur and methionine. Dziewiatkowski (116), using radioactive sulfur, found that small quantities of sulfide sulfur were converted by the rat into cystine sulfur. Leuthardt & Fahrländer (117) presented data

to show that methionine or cysteine inhibited urea formation in liver slices.

The mechanism of formation of amino acids from pyruvate and ammonia has been studied by Kritzmann (118) in surviving rat liver tissues. He concluded that the synthesis of amino acids from ammonia and pyruvate is not a simple reductive or acetylating amination of pyruvic acid, but is a complicated process involving the formation of oxaloacetate through condensation of pyruvate with carbon dioxide, followed, possibly, by the production of α -ketoglutaric acid by way of the tricarboxylic acid cycle of Krebs. Alanine is formed by transamination between aspartic acid (from oxaloacetic acid) or glutamic acid (from α -ketoglutaric acid) and pyruvic acid. Bloch & Rittenberg (119) fed deuteroacetyl derivatives of a number of amino acids to rats, together with foreign amines which are excreted as acetyl derivatives. Their results suggested that transfer of acetyl groups between two α -amino acids may take place. In their studies on the metabolism of L-histidine Tesar & Rittenberg (120) found the highest concentration of N¹⁵ from isotopic histidine in the blood plasma and liver proteins of rats fed this amino acid. There was no evidence in their results that histidine acted as a precursor of glutamic acid, arginine, creatine, or purines.

In a study of the incorporation of radioactive carbon dioxide, Anfinsen and co-workers (121) found that essentially all of the radioactivity assimilated by the liver proteins is located in aspartic and glutamic acids. Darling (122), in a kinetic and thermodynamic investigation on the transamination process, using transaminase, studied the reaction: L-glutamic acid + pyruvic acid \rightleftharpoons α -ketoglutaric acid + L-alanine. The equilibrium constant, temperature coefficient, energy of activation, heat of reaction, free energy change, and the free energy of the divalent α -ketoglutaric acid ion and the monovalent pyruvic acid ion were calculated. The conversion of citrulline to arginine by transamination with glutamic acid in tissue slices and in homogenates has been studied by Cohen & Hayano (123, 124, 125). Their data indicate that the transamination reaction is associated with a high energy phosphate donor system. Glutamic acid appears to be an obligatory intermediate for the introduction of ammonia at the citrulline \rightarrow arginine step of the urea cycle. Transamination is probably limited chiefly to liver and kidney, the lack of a highly active arginase

system in the kidney preventing that organ from contributing significant amounts of urea.

Cohen & McGilvery (126, 127) have sought a simple model for peptide bond synthesis, presenting data on the formation of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine by rat liver slices or homogenates. The reaction does not proceed anaerobically nor in the presence of oxidative inhibition. The energy of formation of the peptide bond comes from oxidative processes and probably proceeds through a high energy phosphorylated intermediate. That formation of peptides may play an important role in the synthesis of proteins is indicated by the work of Friedberg, Winnick & Greenberg (128) who demonstrated the formation of a C^{14} labeled dipeptide, leucylglycine, following the administration of radioactive glycine and ordinary L-leucine. The work of Friedberg (129) with labeled isotopes emphasized the activity of the intestinal wall in protein synthesis.

The entrance of amino acids into the carbohydrate path is illustrated by the work of Schofield & Lewis (130) who demonstrated an increase of liver glycogen in rats after the absorption of the following amino acids which are listed in the descending order of their effect on the production of glycogen: DL- and D-alanine, DL-serine, L-alanine, and DL-isoserine. Kaplanskii & Shmerling (131) studied the influence of acetic, acetoacetic, and β -hydroxybutyric acids on the synthesis of amino acids in liver and kidney slices, and they have reported on the formation of a new type of amino acid, possibly β -aminobutyric acid.

Lotspeich & Pitts (132), in their studies on the role of amino acids in the renal tubular secretion of ammonia, concluded that renal amino acid oxidases are concerned with the synthesis of ammonia by the kidney, this synthesis playing an important role in the regulation of acid-base balance. The results of work by Friedman (133) suggested that feeding glycine increased urinary uric acid in man and in the rat by affecting the amount of uric acid reabsorbed by the renal tubule.

Shemin & Rittenberg (134), using N^{15} , demonstrated that glycine nitrogen is used directly for the synthesis of protoporphyrin in the rat, while the nitrogen of proline, glutamic acid, leucine, and ammonia is used indirectly, presumably by way of glycine. These authors suggest that porphyrins are biologically synthesized through the condensation of glycine with a β -keto aldehyde formed

in part, at least, from acetic acid. Their studies (135) on isotope concentrations in the heme of human red blood cells demonstrated that glycine is the nitrogenous precursor of protoporphyrin and that the erythrocyte has a definite life span of about 127 days. Using these same techniques, Shemin (136) has found that a considerable fraction of L-serine is converted, with utilization of its carbon and nitrogen, directly into glycine in the rat and guinea pig. This conversion takes place by splitting off of the β -carbon atom. Shemin & Rittenberg (137) have demonstrated, in man, that glycine contributes nitrogen to position 7 of uric acid, and that, probably, the carbon atom 5 is derived from the α -carbon of this amino acid. Sonne, Buchanan & Delluva (138) and Buchanan & Sonne (139), in their studies on uric acid formation in the pigeon, found that glycine, or a metabolic derivative of it, is probably the precursor of carbon atoms 4 and 5 of uric acid. The data obtained also lead to the conclusion that carbon dioxide is the precursor of carbon atom 6 of uric acid, and that the carboxyl carbon of acetate is the precursor of carbons 2 and 8.

Borsook & Dubnoff (140) have made an analysis of the hydrolysis of phosphocreatine and the origin of urinary creatinine. They found that the following reactions take place in aqueous solutions of phosphocreatine at 38° C., all of the reactions being first order and independent of each other: (a) phosphocreatine \rightarrow creatinine + inorganic phosphate; (b) phosphocreatine \rightarrow creatine + inorganic phosphate; (c) creatine \rightarrow creatinine. Reaction *a* is three to four times faster than *c*. In a study of labile methyl groups needed for creatine formation and for lipotropism, Tidwell (141) found no decrease in the excretion of creatine by rats on a diet deficient in labile methyl groups.

DEPLETION IN PROTEINS

Some effects of the lack of essential amino acids in the diet are revealed by studies on low protein diets. Allison and associates (8) have demonstrated, for example, the marked decrease in total circulating plasma albumin which accompanies feeding a protein-free diet to dogs. The plasma volume decreased and the extracellular fluid increased as the plasma albumin fell, resulting in a nutritional edema that was corrected rapidly by repletion with proteins. The reduction in plasma proteins represented a decrease in protein stores which was reflected by a fall in the excretion of

urinary nitrogen, particularly urea and ammonia (8). Allison, Anderson & Seeley (6) presented data similar to those of Zeldis *et al.* (142) to show that the albumin-globulin ratios, determined by salt fractionation, are greater than those determined through electrophoresis. Since the correlation between the ratios determined by the two methods was regular and essentially linear, either method revealed relatively similar changes. Chow (143) has reviewed changes in electrophoretic patterns of plasma albumin and globulins, associated with protein depletion in the dog, and has presented data showing similar changes in blood proteins of man suffering from malnutrition, tuberculosis, or cancer. Thermal injury in rats was found by Gjessing & Chanutin (144) to cause an increase in the concentration of α - and β -globulins and a decrease in γ -globulin and albumin, changes from normal similar to those which Chow (143) found associated with depletion in proteins. Accompanying an increase in the lipid fraction of the blood in animals depleted in proteins is a decrease in liver function (145, 146) and the production of a fatty liver. Li & Freeman (146, 147), for example, found that a fatty liver was produced in dogs maintained for ten to sixteen weeks on a 33 per cent fat, protein-deficient diet. They found, too, that the incidence of "peptic" ulcers was high in protein-deficient dogs (148). Kosterlitz (149) has reported that

losses in protein, phospholipin and nucleic acid during fasting, and in protein deficiency, are due to a loss of liver cytoplasm, both particulate and interparticulate structures being affected.

That protein-deficient diets will produce bone atrophy in the mature rat was demonstrated by Armstrong & Estremera (150).

Whipple, Robscheit-Robbins & Miller (1) have summarized their concepts of the dynamic equilibrium between the blood proteins and tissue proteins. Their recent studies (151) have been conducted on doubly depleted dogs. The double depletion is produced by sustained bleeding of animals fed a protein-free, or low nitrogen, diet with adequate iron. Thus the reserve stores of blood protein-producing materials are reduced, levels of 6 to 8 gm. per cent of hemoglobin, and 4 to 5 per cent of plasma protein being maintained in the depleted animal. New hemoglobin in these dogs may be derived in part from plasma proteins but hemoglobin contributes to the "protein pool" for exchanges only after the red cell is destroyed (1). The concept that plasma proteins may disappear

from the blood under protein need, apparently without being broken down to amino acids, has been emphasized recently by Bock (152). Further studies by Whipple, Miller & Robscheit-Robbins (7) have shown that doubly depleted dogs will continue to produce much plasma and hemoglobin for many weeks while being fed a low protein or protein-free diet with abundant iron. Thus the blood proteins take priority under these conditions over other tissue proteins. This heavy demand on body proteins to form blood proteins does not bring about a "premortal rise" in urinary nitrogen: the excretion remains low and body nitrogen is conserved. The authors suggest that "premortal rise," in many experiments, may be associated with a terminal infection leading to catabolism of tissue nitrogen. Continuing their studies on the effect of a diet low in protein, Orten & Keller (153) have reported that porphyrin excretion was reduced below normal in rats deficient in proteins, a chronic anemia developing.

REPLETION IN PROTEINS

As the protein stores are repleted, the urinary nitrogen excretion increases. Allison and associates (8) have shown that this is the result of a decrease in the utilization of dietary protein, and of an increase in the excretion of nitrogen from body stores. The greater retention of nitrogen by depleted than by normal dogs, reported by these authors, suggests that there is a different type of internal supplementation in the depleted than in the normal animal. Whipple, Robscheit-Robbins & Miller (1) found that a relatively incomplete protein, like globin, can contribute effectively to the protein stores of the doubly depleted dog, and they suggest that globin must be internally supplemented. They found that these depleted animals will use efficiently a variety of proteins, digests, and the growth mixture (Rose) of pure amino acids. The capacity of dietary nitrogen to replete in these experiments is measured in terms of a production ratio which is the ratio between protein output and intake. Relative production of hemoglobin, with respect to plasma protein, is expressed as a ratio between plasma protein and hemoglobin. The utilization of dog hemoglobin by these doubly depleted animals was improved by methionine but not by isoleucine (154). Further observations by Miller & Alling (155) demonstrated that when DL-isoleucine was added with methionine, or with methionine and cystine, the utilization of

parenterally given hemoglobin was improved. Possibly egg protein favored slightly the production of plasma protein, while whole beef plasma (oral) favored the production of hemoglobin (151). The average production ratio for the ten essential amino acids (Rose) was 19 (156). When one of the essential amino acids was removed the ratio rose to 25. On a good dietary protein the ratio was 15. Whipple and associates suggest that good dietary proteins like egg albumin and lactalbumin are utilized to replete protein stores in organs and tissues, this repletion being reflected in a moderate way in the blood proteins. The amino acid mixtures, on the other hand, with or without one of the ten essentials, caused loss of weight and from this loss came materials which accelerated the formation of blood proteins more than when the animal was receiving a good dietary protein. They found that methionine, threonine, phenylalanine, and tryptophane, when eliminated singly from the growth mixtures of amino acids, resulted in a sharp rise in urinary nitrogen, a rise which was corrected when the amino acid was replaced in the mixture. Tryptophane, and to a lesser extent, phenylalanine and threonine, when returned to the amino acid mixture, were associated with a preponderance of plasma protein output over the hemoglobin output. Arginine, lysine, and histidine, on the other hand, were associated with hemoglobin output. None of the amino acid mixtures, whether they contained the ten essentials or not, prevented weight loss in the doubly depleted dog. A good dietary protein such as casein, lactalbumin, whole egg, or liver protein did prevent this loss in weight. Miller, Robschey-Robbins & Whipple (157) suggest that some unidentified substances may be present in the dietary proteins and absent from the amino acid mixture which could be responsible for maintenance of weight in the doubly depleted dog.

Frazier *et al.* (61) studied the dietary utilization of mixtures of purified amino acids in protein depleted adult albino rats. These studies demonstrated that the protein depleted rats gained weight rapidly, and maintained good appetites, when fed the nine essentials, arginine being dispensable. Thus, the same nine amino acids, essential for growth, were also essential for repletion of these depleted rats. Omission singly of any one of the nine essentials from the diet led to a marked loss of weight and a prompt loss of appetite, the daily food consumption being reduced. Good appetite and weight gains were maintained when lysine was administered

parenterally and the other acids were consumed orally. When, however, the lysine was replaced by a salt solution both the weight and appetite of the animal declined. Benditt *et al.* (158) found that the nine amino acids, essential for appetite maintenance and weight gain, were also indispensable in the hypoproteinemic rat for construction of serum protein and erythrocytes.

The formation of specific plasma proteins has been studied by Allison, Anderson & Seeley (6) and Chow (143) who found that repletion with a casein hydrolysate favored the formation of the β -globulin fraction, while repletion with a lactalbumin hydrolysate favored the formation of plasma albumin. These studies involved the production of a standard protein-depleted dog followed by a repletion period of thirty days, sufficient time for the best dietary proteins to return the dog to normal (6). Gurd, Vars & Ravdin (159) have shown a close correlation between regeneration of liver protein and the amount of nitrogen retained in the rat, irrespective of nitrogen balance. Vars & Gurd (160) found a correlation between the nutritive value of the dietary protein and the regeneration of liver protein.

Parenteral administration.—Modern protein therapy requires the parenteral administration of amino acid mixtures or protein hydrolysates. Frost, Risser and associates (161 to 165) have applied nitrogen balance techniques to dogs for the evaluation of casein and fibrin hydrolysates (variously supplemented by essential amino acids). They obtained data, which could be described by equation I, permitting them to calculate nitrogen balance indexes. They present evidence that peptides are utilized for anabolic processes. Kade *et al.* (166) maintained nitrogen balance for twenty-one to thirty-five days in dogs fed an acid hydrolysate of casein fortified with tryptophane. Cox & Mueller (167) compared the relative efficiency of an enzymatic casein hydrolysate and two mixtures of crystalline amino acids in maintaining nitrogen equilibrium in protein-depleted dogs. Allison, Seeley & Ferguson (12) developed a technique for the evaluation of hydrolysates fed intravenously to dogs. They point out that nitrogen balance indexes were higher in oral than in intravenously fed hydrolysates. Silber *et al.* (168) have made a thorough study of the maintenance of dogs on a diet containing amino acids fed orally or intravenously. Their amino acid mixture favored, more than casein, the formation of liver protein. Intravenously administered amino acids

were found by Friedberg & Greenberg (169) to be rapidly removed from the plasma and concentrated in liver and kidney tissues. Parenteral alimentation with special reference to proteins and amino acids has been critically studied and reviewed by Elman (170, 171).

Protein therapy.—Space does not permit a survey of the important field of protein therapy, but this review of protein metabolism is made more complete by recording a number of papers that deal with the subject (46, 170 to 179).

HORMONES IN NITROGEN METABOLISM

More evidence has accumulated to demonstrate the important role played by hormones in protein metabolism. Friedberg & Greenberg (180) have shown that insulin, epinephrine, estrogen, hypophysectomy, thiouracil treatment, and thyroidectomy decreased, while thyroxine and lipo-adrenal cortex extract and protein starvation increased, blood amino acids. Li & Reinhardt (181) found that there was a rise in plasma albumin concentration in hypophysectomized rats treated with either growth or adreno-corticotrophic hormone but that the latter hormone did not cause a rise in the concentration of the globulin fractions of the plasma or lymph. Eisen and associates (182) found no evidence that adrenal cortical activity is essential for the formation of antibodies and γ -globulin. That the adrenal cortical hormones may be necessary for, but not the specific cause of, the increase in urinary protein nitrogen following fractures in rats has been demonstrated by the work of Ingle, Ward & Kuizenga (183). The effect of a high protein diet on plasma protein concentrations and organ weight, a diet which was thought might produce adrenal hypertrophy, was studied by Leathem (184); there was a modest increase in adrenal weight, but no indication of increased functional activity. Leathem (185) and Leathem & Seeley (186) found an increase in plasma globulin, total plasma protein, and nonprotein nitrogen in rats fed thiourea or thiouracil. They reported that thiouracil increased the size of the liver without changing its protein concentration. In a comparison study Leathem (187) showed that the feeding of thiourea caused hypertrophy of the thyroid, but did not influence other organ weight/body weight ratios in spite of a body weight loss associated with reduction in food intake. Gol'dshtein *et al.* (188) put forth the view that the thyroid hormone may be involved

in a rupture of a thio ether linkage in proteins, liberating sulfhydryl groups, the hormones participating in the process of transmethylation when methionine is involved in this rupture. Gordan *et al.* (189) demonstrated that the injection of growth hormone in rats fed a 6 per cent casein diet did not increase weight, but did increase nitrogen retention. The addition of methionine made this casein diet capable of supporting the rapid growth associated with the growth hormone. Samuels (190) suggested

that the variation between different proteins or amino acid mixtures in their ability to be used anabolically is in part due to their influence on the metabolism of the pituitary gland rather than on their influence on the body cells in general.

These and other studies emphasize the recent trend toward integrating protein and amino acid metabolism with other metabolic processes, an integration which is essential to a full understanding of the living machine.

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THE METABOLISM OF DRUGS AND TOXIC SUBSTANCES

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The general subject matter of the present article has been considered in previous reviews under the title of "Detoxication Mechanisms." The term "detoxication" was originally employed on the assumption that the metabolism of a drug or toxic substance followed special pathways and proceeded in the direction of the formation of less toxic compounds (1). In 1941 Stekol (2) pointed out that such an assumption was unwarranted in view of the lack of information concerning the relative toxicities of parent substances and their derivatives and in view of the findings that the metabolic mechanisms were usually no different from those operating in the normal transformation of food stuffs. Handler & Perlzweig (3) and Welch & Bueding (4) re-emphasized this approach and cited instances in which the conversion products had been revealed to be more toxic than the parent substances. It has also been shown that some substances do not exert their characteristic toxic or pharmacological activities until they have undergone a metabolic change.

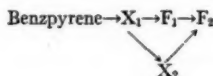
There is still another way in which the term "detoxication" has proven inappropriate. The literature reveals an increasing preoccupation with the metabolism of drugs or substances which are of therapeutic importance. This seems quite fitting, for there is no essential difference in this respect between drugs and toxic substances. Drugs in doses greater than those employed for therapeutic effects become toxic substances and, similarly, compounds usually considered as toxic have at times been employed in smaller doses for therapeutic purposes.

In view of these considerations, we shall, as the title of our review indicates, concern ourselves with the metabolism of drugs and toxic substances with attention to but without bias regarding the relative toxicity or pharmacological effectiveness of the original and derived substances. The subject may be approached either from the viewpoint of a chemical or pharmacological classification

or from the viewpoint of the types of metabolic mechanisms which are involved. Although earlier work attempted to establish the existence of such mechanisms as oxidation, reduction, conjugation, methylation, etc., in the transformation of drugs and toxic substances, recent investigations have tended to take their existence for granted and to describe in detail the sequence of these mechanisms in the metabolism of various groups of substances such as steroids, hydrocarbons, pressor amines or of compounds such as alcohol or salicylic acid (3,5). We shall continue and indeed emphasize this trend. However, in the latter part of the review we shall consider, in a more general way, certain types of metabolic mechanisms which have been studied during the past few years. These are: (a) sulfhydryl combination, (b) glucuronide and hippuric acid formation, and (c) the phenomenon of metabolic adaptation.

The material considered in the present review constitutes a selection of articles which have appeared between October, 1944, and November, 1947.

Hydrocarbons.—The metabolism of carcinogenic hydrocarbons is of obvious importance. Earlier studies of the metabolism of 3,4-benzpyrene in which it had been shown that the stools of mice and rats probably contained 8-hydroxy-3,4-benzpyrene and 3,4-benzpyrene-5,8-quinone (6, 7, 8) have now been followed by more detailed investigations. Weigert & Mottram (9), employing fluorescence and absorption spectra and chromatographic analyses, have obtained indication for the existence of the following metabolic sequence in mice:



X_1 represents the compound, 8(OR₁)-9(OH)-8,9-dihydro-3,4-benzpyrene; X_2 represents 8(OR₁)-9(OR₂)-8,9-dihydro-3,4-benzpyrene and F_1 is 8(OR₁)-3,4-benzpyrene. F_2 is 8-hydroxy-3,4-benzpyrene which, as mentioned above, had previously been described as the end product of metabolism. The nature of R_1 and R_2 is not yet established but it is postulated that they are derived from cellular material. Berenblum & Schoental (10) present a somewhat different course for the metabolism of this compound in the rabbit; 8- and 10-benzpyrenols and 5,8- and 5,10-benzpyrenequinones are found in the feces.

These observations are in accord with Fieser's perhydroxylation theory of polynuclear hydrocarbon oxidation (11). According to this formulation, two hydroxyl groups hydroxylate a double bond at a site other than the one of special reactivity. Water splits off from the intermediate dihydro-dihydroxy compound, leaving a phenolic group. In the benzpyrene molecule, the most reactive position is at carbon 5. The existence of this intermediate compound is supported by the observation made in 1935 by Boyland & Levi (12) that 1,2-dihydroxy-1,2-dihydroanthracene was found in the urine of rabbits and rats which were fed anthracene. Young (13) has now reported the isolation of 1,2-dihydroxy-1,2-dihydronaphthalene from the urine of rats fed or subcutaneously injected with naphthalene. About 4.5 to 10.5 per cent of the administered naphthalene was excreted as dihydroxy-dihydro derivative and about 12 per cent as 1-naphthyl-L-mercapturic acid. Booth & Boyland (14) have isolated the same compound from rabbits; the melting point, however, was somewhat lower and the compound was probably a racemate. Berenblum & Schoental (15) observed that when chrysene was administered to rats, the introduction of a phenolic group occurred, not at the most reactive position 2, but at carbon 3. The introduction of a phenolic group into 2-acetamidofluorene occurs at carbon 7 (16).

Pressor amines.—The *in vivo* and *in vitro* metabolism of the pressor amines has recently been reviewed by Hartung (5); the roles of amine oxidase, phenolase, cytochrome and the ascorbic dehydroascorbic acid systems have been outlined, with particular attention being given to the metabolism of epinephrine. The relationship between molecular structure and amine oxidase action has been explored further by Snyder, Goetze & Oberst (17) and, although the principal conclusions of Beyer & Morrison (18) have been confirmed, others have been slightly modified. Thus, in support of Beyer & Morrison's formulation that substitution of a single methyl group on the β -carbon did not appreciably alter the rate of deamination, the uptake of oxygen was found to be 1.1 mM per mM of β -methyl- β -phenylethylamine and about 0.9 mM per mM of phenylethylamine. Again, in accordance with the formulation that secondary methylamino compounds were more rapidly deaminated, provided that the alkyl group was not too long, the uptake of oxygen was found to be about 1.6 mM per mM of N-methyl- β -methyl- β -phenylethylamine. However, in contrast

to Beyer and Morrison's finding that a hydroxyl group on the *p*-position of the benzene nucleus increased the rate of deamination markedly, Snyder, Goetze & Oberst (17) found that the uptake of oxygen was about equal for β -phenylethylamine and tyramine. Compounds with a methyl group in the α -position or with two substituent groups in the β -position were not readily metabolized. Compounds subject to deamination were excreted to only a slight extent in the urine, 7 per cent or less of the administered dose, whereas compounds which were not attacked, such as amphetamine or propadrine, showed large excretions, 15 to 43 per cent of the administered dose. Harris, Searle & Ivy (19) noted that on a continuous daily intake of amphetamine the excretion of this compound remained constant at about 50 per cent of the ingested dose, thus indicating a constant rate of catabolism. Similar relationships between molecular structure and amine oxidase action have been observed for the cyclohexylethylamines (20). Randall (21) has observed that 2-hydroxyphenylethylamines are oxidized less rapidly than the 3 and 4 position isomers; the converse, however, holds for the methoxy derivatives.

There has been some disagreement as to the factors involved in the action of liver amine oxidase, particularly concerning the uptake of oxygen under various experimental conditions (22 to 26). Govier *et al.* (27) found that, with tyramine as substrate, the oxygen uptake was increased in the presence of cytochrome-*c*, coenzyme I, pyruvate or methylene blue; they suggested the existence of a coupled reaction between a dehydrogenase ("amine oxidase") with tyramine as substrate and the lactic dehydrogenase in the presence of pyruvate. The cytochrome-cytochrome oxidase system serves as a final path to atmospheric oxygen. This reaction is then followed by the oxidation of *p*-hydroxyphenyl-acetaldehyde, resulting from the deamination of the tyramine, to *p*-hydroxyphenyl-acetic acid. It is possible that this latter reaction is catalyzed by xanthine aldehyde oxidase.

Phenols.—Compounds with a phenolic group interact both with sulphuric and glucuronic acids. De Meio & Arnolt (28) found that in the cat the liver, kidney and spleen were the tissues which showed most active conjugation *in vitro*. Such conjugation is exhibited by tissue slices but not by tissue brei. Conjugation is inhibited by anaerobiosis, cyanide ion or monoiodoacetate ion. Inhibition in the former two instances indicates the necessity of

an oxidative coupled reaction. Monoiodoacetate ion, in 0.02 *M* concentration, inhibits phenol conjugation by liver slices of the rat (Vanderbilt strain), guinea pig, dog and cat; addition of glucuronic acid does not re-establish conjugation in these instances, although it has been reported to have such an effect in another strain of rats (29). Phenol conjugation is also inhibited by azide, octyl alcohol, oxalate ion, bisulfite and fluoride (29).

Liver slices of the dog, guinea pig, the Vanderbilt strain of rats and the *Rana pipiens* frog show no or very slight conjugation in the absence of sulfate ion, but liver slices of the cat, another strain of rats (Rosario Medical School strain), the *Rana catesbiana* frog and the chicken show good conjugation (30). The presence of sulfate ion establishes or increases conjugation in all of these species, with the exception of the chicken. Liver slices of scorbutic guinea pigs conjugate phenol to the same extent as liver slices from normal guinea pigs (31).

Aromatic amines.—It has long been known that aniline is converted to *p*-aminophenol. Rubanovskaya (32, 33) has reported that this oxidation is accomplished chiefly by the kidneys and that ascorbic acid increases the survival of rabbits and rats poisoned with aniline, presumably by accelerating the conversion of aniline. The controversies concerning the metabolism of the substituted aniline, acetanilid, have been reviewed by Greenberg & Lester (34). These workers have established that, in man, 70 to 90 per cent of the administered acetanilid appears in the urine as conjugated *p*-aminophenol (35, 36). Of the total *p*-aminophenol excreted, 4 per cent appears as the *N*-acetyl conjugate and 96 per cent as the hydroxy conjugates which, according to previous observations, are chiefly in the form of the sulfate or glucuronide. The urine also contains conjugated aniline, corresponding to about 0.2 to 0.6 per cent, and azo compounds corresponding to 0.5 per cent of the administered acetanilid. *N*-acetyl-*p*-aminophenol and its hydroxy conjugates are present in considerable concentrations in the plasma. It would therefore appear that the major metabolism of acetanilid consists, first in the oxidation at the *para* position to form *N*-acetyl-*p*-aminophenol and, secondly, in the conjugation of the phenol group.

Sulfonamides.—Because of their therapeutic significance, the metabolism of the sulfonamide drugs has claimed particular attention. Most of the earlier studies in this field have concerned

themselves with the extent of excretion of the sulfonamides and with the degree of conjugation. For example, among such studies is that of Strauss and co-workers (37) in which it was shown that after a single intravenous dose of 5 gm. of sulfanilamide, sodium sulfapyridine or sodium sulfathiazole in man, between 80 and 100 per cent of the injected drug was excreted in the urine in seventy-two hours and that up to 90 per cent of the excreted drug was recoverable in conjugated form. Reinhold, Flippin & Schwarz (38) found that about 97 per cent of intravenously injected sulfathiazole was excreted in the urine in twenty-four hours. More recently, with the advent of sulfonamides for use in intestinal infections, the relative excretion in urine and feces has become of interest. Thus, Meads & Finland (39) have reported that orally administered sulfathiadiazole is recoverable, on the average, to the extent of about 83 per cent in the urine and of 4 to 8 per cent in the feces. In contrast, orally administered sulfacarboxythiazole is excreted almost entirely in the stools (83 to 92 per cent), and only to the extent of 3 to 7 per cent in the urine (40). Taylor, Snyder & Oberst (41) have recently studied a series of hitherto unreported sulfonamides which are excreted in the feces to the extent of about 75 per cent of the administered dose.

It should be emphasized that the rather considerable urinary or fecal excretions which have been reported for most of the sulfonamides do not necessarily represent the unaltered, administered compound. The Bratton & Marshall procedure (42), employed in the quantitative determination of the sulfonamides, consists in a diazotization of an aryl amino group with a subsequent coupling to yield a colored compound. Accordingly, any diazotizable intermediate or end product of sulfonamide metabolism is also determined by this test. Scudi (43, 44) as well as Weber, Lalich & Major (45) has obtained evidence indicating that in the dog sulfapyridine is metabolized to a compound which contains a hydroxyl group in the pyridine ring, probably in the 3 position, and which is conjugated with glucuronic acid in that position. Oxidation has also been demonstrated in other sulfonamides. In the rabbit and man, sulfanilamide is oxidized at the 3 position to yield 3-hydroxy-4-aminobenzenesulfonamide (46, 47). The amount oxidized is small; in the rabbit about 7 to 9 per cent goes through this pathway and is excreted as ethereal sulfate. No *p*-hydroxyl-

aminobenzenesulfonamide or azoxybenzene-4,4'-disulfonamide is found in rabbit urine.

It would appear, then, that the two chief pathways in the metabolism of the sulfonamides are an acetylation of the amino group attached to the benzene nucleus and an oxidation either in the benzene nucleus or in the substituent group attached to the sulfone radical. This oxidation results in the formation of a phenolic group through which conjugation occurs with sulfuric or glucuronic acid. The role of these two pathways varies with the particular sulfonamide and the species. For example, in a study of sulfonamide excretion in man, Gilligan (48) found that sulfadiazine and sulfathiazole were acetylated least and sulfamethazine most. Glucuronide excretion was greatly increased when sulfapyridine, sulfamerazine or sulfamethazine were administered, but no increase occurred with sulfanilamide, sulfadiazine or sulfapyrazine. There was no consistent increase in the ethereal sulfate excretion with any of these drugs. Beyer and his associates (49) observed that in the rat sulfathiazole was acetylated much more rapidly than the pyrimidine derivatives.

The mechanism of acetylation has been investigated further by Lipmann (50). Pigeon liver homogenates acetylated sulfanilamide very actively, and the degree of acetylation was enhanced by acetate and, to a lesser degree, by other metabolites such as pyruvate and acetoacetate. Under anaerobic conditions, acetylation was reduced considerably, but was restored in the presence of adenylyl pyrophosphate. Lipmann suggests that acetate and sulfanilamide form a complex with adenylylpyrophosphate, whether the latter be added or formed during respiration. The complex then breaks up into acetylsulfanilamide, adenylic acid and inorganic phosphate. The enzyme system which mediates the formation of this complex is freely soluble, may be separated from particulate matter and is dependent, for its activity, on a heat stable coenzyme which appears to be a pantothenic acid derivative and which also plays a role in the acetylation of choline (51, 52).

The metabolism of two sulfonamides in which the amino group is separated from the benzene nucleus by a methyl radical is of some interest. These compounds are *p*-aminomethylbenzenesulfonamide, $\text{NH}_2 \cdot \text{CH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_2 \cdot \text{NH}_2$ also known as ambamide or marfanil, and *p*-methylsulfonylbenzylamine hydrochloride (V-355),

$\text{HCl} \cdot \text{NH}_2 \cdot \text{CH}_2\text{C}_6\text{H}_5 \cdot \text{SO}_2 \cdot \text{CH}_3$. The first of these, ambamide, has considerable immediate *in vitro* antibacterial activity which, unlike that of the sulfonamides which possess an aromatic amino group, is not inhibited by *p*-aminobenzoic acid. It has an *in vivo* effect when injected at the site of infection, but none when introduced systemically. These properties indicate that the compound is destroyed rapidly when introduced into the organism. Beyer & Govier (53) and Blaschko & Duthie (54) have shown that these two sulfonamides are acted upon by amine oxidase. The Bernheims have found that rat liver, in contrast to rat kidney and guinea pig liver and kidney, does not inactivate ambamide, although rat liver presumably contains more amine oxidase than does rat kidney (55). As the Bernheims point out, the possibility exists that there are several types of amine inactivating enzymes and that the one acting on ambamide differs from those acting on other amines (56). Hartles & Williams (57) report that when ambamide is fed to the rabbit, it is rapidly oxidized and is excreted in the urine as *p*-carboxybenzene-sulfonamide; as much as 84 per cent of the ambamide can be accounted for in this manner. There is no excess excretion of ethereal sulfate, glycine conjugate or glucuronide, and no nuclear oxidation or acetylation. V-355 is similarly metabolized. These sulfonamides, then, are metabolized in a similar manner as the pressor amines; deamination occurs and the methylene grouping is oxidized to carboxyl.

Steroids.—Our knowledge of the *in vivo* transformations of steroids has continued to be augmented through the isolation of urinary excretion products. Following the administration of 1090 mg. of dehydroisoandrosterone over a period of sixteen days to a man with anterior pituitary insufficiency, Mason & Kepler (58) recovered the following products from the urine in purified form: 79 mg. of dehydroisoandrosterone, 130 mg. of androsterone, 73 mg. of etiocholan-3 (α)-ol-17-one and 6.5 mg. of Δ^5 -androstene-3(β), 17(α)-diol. The appearance of androsterone suggested that either androstane-3,17-dione or Δ^4 -androstene, 3,17-dione was an intermediate. Either the latter compound or etiocholane-3,17-dione might be a further intermediate in the formation of etiocholan-3(α)-ol-17-one. The substance Δ^5 -androstene-3(β), 17(α)-diol is a reduction product of dehydroisoandrosterone. Bloch (59) has reported that the administration of deuteriocholesterol re-

sulted in the production of deuteriopregnanediol-3(α), 20(α). Mason & Kepler (60) have found that the administration of dehydroisoandrosterone to two cases of Addison's disease resulted in the excretion of androsterone, etiocholanolone, Δ^5 -androstene-3(β), 16, 17-triol and a nonketone.¹

There has, however, been increasing emphasis on the *in vitro* analysis of the mechanisms involved in steroid metabolism. In 1934 Zondek (61) had demonstrated that the brei of liver but not of other tissues exerted a destructive effect on estrone. Since then a number of workers have confirmed his observation and, in addition, have shown that this inactivation also occurs *in vivo* (62, 63). More recently, Nielsen, Pederson-Bjergaard & Tønnesen (64), employing the vaginal cornification test in spayed rats, have reported that stilbestrol and dienestrol administered either orally or parenterally are not inactivated by liver. Estrone, estradiol and hexestrol are inactivated after oral administration and estriol is inactivated whether given orally or parenterally. Progesterone (65) and desoxycorticosterone (66) are also inactivated to some extent by the liver and progesterone counteracts the *in vivo* inactivation of estradiol by the liver in rats (67).

Stilbestrol is destroyed in the presence of liver slices, and this disappearance may be explained on the basis of the oxidation of this compound as well as by conjugation with sulfate and glucuronide (68). Samuels, McCaulay & Sellers (69) have found that homogenized liver tissue from the rat, mouse, rabbit and man destroys testosterone, whereas tissues from secondary sex organs do not exert this effect. The destruction is dependent upon an oxidative enzyme system, as evidenced by the findings that the destruction is inhibited in a nitrogen atmosphere or in the presence of any of the following substances: 0.01 *M* cyanide, 0.05 *M* iodoacetate, 0.05 *M* fluoride or 0.067 *M* malonate. The nature of the chemical reactions involved in the conversion of testosterone is shown by the work of Clark & Kochakian (70). They incubated testosterone with rabbit liver slices and were able to isolate Δ^4 -androstenedione-3,17 and *cis*-testosterone. It would appear that the 17 α -hydroxyl group is readily oxidized to a ketone group which in turn may be reduced to the 17 α -hydroxyl group and possibly also to a 17 β -

¹ The steroid nomenclature given is that used by the various authors.

hydroxyl group. Lehninger & Scott (71) have observed that estrone and estradiol are adsorbed by human prostate slices but that no metabolic reactions ensue as a result of this adsorption.

Fishman (72) has continued his studies on β -glucuronidase and has observed that, following administration of estrogens, including stilbestrol, the activity of this enzyme is increased in the uterus and vagina, but not in the liver, kidney, spleen or blood. This investigator had previously observed that the administration of glucuronideogenic drugs like borneol or menthol led to the converse situation, namely, an increase in the glucuronidase content of the liver, kidney and spleen, but no change in that of the uterus (73). It is postulated that the increase in uterine β -glucuronidase observed after administration of estrogens is an adaptive process which plays a role in the synthesis of the glucuronides and hence in the metabolism of the estrogens. This aspect will be discussed more fully later on in this review.

Antimalarials.—The prevalence of malaria in areas in which our troops fought during the recent war and the shortage of quinine stimulated fundamental studies into the character of the metabolism of antimalarial drugs. Kelsey *et al.* (74) gave considerable impetus to this subject by isolating the metabolic product formed upon incubation of quinine with rabbit liver. Mead & Koepfli (75) identified this substance as an oxidation product in which the hydrogen atom in position 2 had been replaced by an hydroxyl group to yield quinine carbostyryl. It has been shown that this type of oxidation results in a reduction of antimalarial activity (76).

Kelsey & Oldham (77) found that quinine was destroyed actively by rabbit tissues, particularly by the liver. Cat and guinea pig livers were also active, whereas those of the monkey, pig, steer, chicken, dog and frog showed little or no activity. That the liver is also an important factor in the metabolism of quinine *in vivo* was shown by Anderson, Cornatzer & Andrews (78) who found that partial hepatectomy in the rat caused a marked increase in the urinary excretion of quinine. Because the disappearance of quinine in the presence of liver brei required oxygen and preliminary studies indicated the metabolic product to be the result of oxidation, Kelsey and his co-workers (77) considered the enzyme involved to be quinine oxidase. Knox (79) has studied the properties of this enzyme and found that the reaction which is catalyzed is: qui-

$\text{nine} + \text{H}_2\text{O} = \text{quinine carbostyryl} + \text{H}_2\text{O}_2$. The enzyme has a pH optimum between 7.4 and 7.6 and oxidizes other quinolines as well. Oxidation is prevented by substitution of phenyl or carboxyl group for the alpha hydrogen, and the rate is affected by substituent groups elsewhere. The enzyme appears to be a flavoprotein.

The metabolism of other antimalarials has also attracted considerable attention. Shannon and his co-workers (80, 81) observed that quinacrine (atabrine) is almost completely absorbed from the gastrointestinal tract, that very little of the daily dose is excreted in the urine and that much of the drug is stored in the tissues and subsequently degraded. The formula of quinacrine may be written as 6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine. Hammick & Mason (82) have been able to isolate three degradation products from the urine of humans receiving this drug. In one of these products, the methoxy group on carbon 2 is replaced by a hydroxyl group; in a second substance, the 4-diethylamino-1-methylbutyl group on the amino radical is replaced by hydrogen. In the third product, both of these substitutions occur. The metabolism of the antimalarial drugs has been reviewed in some detail by Blanchard (83).

Xanthines.—Buchanan, Christman & Block (84) have observed that the excretion of true uric acid, as determined by the action of uricase, is not increased significantly after the ingestion of caffeine, theophylline or theobromine. Previous reports of an increased excretion of uric acid after the ingestion of these substances may be ascribed to the formation and excretion of methyluric acids which analyze as uric acid by the phosphotungstic acid reducing method or the silver precipitation method (85). Buchanan, Christman & Block (84) suggest that oxidation at position 8 of the purine structure and demethylation at other positions would yield the various methyluric acids. Administration of theobromine does not increase the phosphotungstic acid reducing materials in the urine, presumably because the methyluric acid which is formed does not exert such a reducing effect. Myers & Hanzal (86) have studied the metabolism of the methylxanthines and the various methyluric acids in some detail. Theophylline and caffeine appear to be quite completely converted to methyluric acids by oxidation in position 8. Since 1,3-dimethyluric acid is only demethylated to a slight extent at position 3, most of the administered theophylline is excreted as 1,3-dimethyluric acid. Since 1,3,7-trimethyluric acid

is partially demethylated at position 7, caffeine is excreted largely as a 1,3-dimethyluric acid. Because 3-methyluric acid appears to be quite completely demethylated, the absence of increased uric acid excretion after the administration of the xanthines is evidence that such demethylation does not occur in the xanthines. Knowledge of the metabolism of theobromine is uncertain because 3,7-dimethyluric acid is not reduced by phosphotungstic acid and other methods for its determination have not yet been developed. The Bernheims (87) have shown that caffeine and theophylline are oxidized *in vitro* by the liver slices of the rat, guinea pig, and rabbit. Kidney slices are without effect. Some evidence was obtained for partial demethylation at position 3 under these circumstances.

Salicylic acid.—The metabolism of salicylic acid has assumed renewed importance because of recent studies on the toxicity of this substance in the course of treatment of rheumatic fever (88, 89, 90). Kapp & Coburn (91) had observed that normal adolescents and adults excrete about 20 per cent of ingested sodium salicylate in unchanged form. Of the remaining 80 per cent, about 55 per cent was excreted as salicylurate and about 25 per cent as glucuronide. Very small amounts, about 4 to 8 per cent, were eliminated as gentisic acid and related compounds. Smull, Wégria & Leland (92) found that lower serum levels were obtained after the simultaneous administration of sodium salicylate and sodium bicarbonate than after the administration of sodium salicylate alone. Smith and his co-workers (93) have recently reported that this was due to the fact that the renal clearance of free salicylate increased rapidly above urinary pH values of 7.0.

Cyanides.—The combination of cyanide ion with cytochrome oxidase, previously demonstrated *in vitro*, has now been shown to hold *in vivo*; in rats injected intraperitoneally with 5 mg. sodium cyanide per kg., the brain cytochrome oxidase activity was reduced to 50 per cent of normal (94). Albaum, Tepperman & Bodansky (95) have studied *in vitro* the competition of methemoglobin and cytochrome oxidase for cyanide ion, in an attempt to elucidate the therapeutic as well as prophylactic efficacy of induced methemoglobinemia in cyanide poisoning (96). The metabolism of cyanogen chloride has been investigated by Aldridge & Evans (97). Blood or red corpuscles convert cyanogen chloride into hydrocyanic acid to the extent of about 30 per cent *in vitro*, and probably somewhat more *in vivo*. This conversion appears to con-

sist of two stages: first, the combination of cyanogen chloride with hemoglobin and secondly, the liberation of hydrocyanic acid from this compound in the presence of reduced glutathione. Cyanogen chloride is ultimately converted to thiocyanate.

Carcinogenic azo dyes.—The metabolism of the carcinogenic azo dyes has been studied further by Miller, Miller & Baumann (98) who showed that, *in vivo*, N,N-dimethyl-*p*-aminoazobenzene (DMB) is demethylated prior to reduction at the azo linkage; the demethylation to *p*-monomethylaminoazobenzene (MMB) is reversible. The noncarcinogenic analogue N,N-diethyl-*p*-aminoazobenzene (DEB) is also dealkylated in the rat (99). Kensler, Magill & Suguira (100) have confirmed the finding that, after oral administration of N,N-dimethyl-*p*-aminoazobenzene (DMB), the chief azo compound found in the tissues is *p*-aminobenzene, although it constitutes only a small portion of the total dye administered. Liver slices do not appear to demethylate these dyes *in vitro*. DMB and AB are destroyed at about the same rate by normal liver. The rate of destruction of DMB by liver tumor slices is about one-third of normal.

Miscellaneous drugs and toxic substances.—Larson, Haag & Finnegan (101) have submitted evidence that the metabolism of nicotine involves a cleavage of the pyrrolidine ring between the nitrogen atom and the 5 position. Mazur (102) has reported that an enzyme capable of hydrolyzing the phosphorus-fluorine linkage of alkyl fluorophosphates is present in the liver, kidney, intestine, plasma and other tissues and that the liver which contains relatively high concentrations of this enzyme plays a significant role in the *in vivo* destruction of the alkyl fluorophosphates. McChesney (103) has found that the oxidation of arsenicals is retarded by ascorbic acid. Bueding & Jolliffe (104) have shown that *in vitro* the metabolism of trinitrotoluene consists in a stepwise reduction of one nitro group with the formation of 4-amino-2,6-dinitrotoluene. This compound as well as 4-hydroxylamino-2,6-dinitrotoluene has been isolated from the urines of rabbits, rats and men exposed to or fed trinitrotoluene (105, 106). Rat urine also yielded 5-nitro-*m*-phenylene diamine. The metabolism of the amoebocide, chiniofon, containing radioactive iodine has been studied (107). About 13 per cent of the drug is absorbed from the intestinal tract and most of this is excreted intact through the urine during the first twelve hours. The Bernheims (108) have found that rat liver slices

cause a marked disappearance of morphine under aerobic conditions. Although it was first judged that this disappearance was due to oxidation, a later study (109) showed that the morphine was conjugated and that autoclaving at 20 lbs. pressure was necessary to hydrolyze the conjugated compound.

Sulfhydryl combination.—It has long been appreciated that the metabolism of certain organic drugs and toxic substances involves combination with sulfhydryl groups present in the proteins of the tissues. Familiar examples of this phenomenon are the formation of the corresponding mercapturic acids when bromobenzene, benzyl chloride or naphthalene are administered. The formation of a mercapturic acid involves the withdrawal of cysteine from body tissues with a resultant inhibition of growth (110). Stekol (111) observed that inhibition by benzyl chloride may be prevented by the simultaneous administration of L-cystine, DL-methionine or DL-homocystine, but not by D-cystine or taurine. Stekol also noted that the possible intermediates in the metabolism of benzyl chloride, namely, benzoic acid and S-benzyl-L-cystine, as well as the final product, N-acetyl-S-benzyl-L-cysteine, do not affect the growth of rats. In this instance metabolism proceeds in the direction of the formation of less toxic substances.

That the toxic actions of heavy metals may be largely due to their combination with the sulfhydryl groups of proteins, particularly the enzyme proteins, was demonstrated by extensive investigations in chemical warfare during World War II. Although previous workers, particularly Voegtlin and his associates (112, 113, 114) had shown that arsenic interacted with sulfhydryl groups, it remained for Stocken & Thompson (115), as a result of their studies with kerateine and lewisite, to point out the probable nature of the chemical grouping: $:C \cdot S \cdot As(R) \cdot S \cdot C:$. In order to find an effective therapeutic agent for lewisite injuries, the British workers sought a sulfhydryl compound which would form a less dissociable complex with the arsinites than did the proteins and which, in this manner, would regenerate the sulfhydryl groups. They found this compound in 2-3-dimercaptopropanol (BAL) (116, 117).

The discovery of BAL has provided us not only with a potent therapeutic agent, but also with a means for studying the interactions of heavy metals with the sulfhydryl groups of proteins. It has been demonstrated that BAL is effective to varying degrees in

cadmium, antimony, bismuth, chromium, nickel and mercury poisoning (118 to 122). Such effectiveness may be explained by the assumption that BAL is capable of forming mercaptides with these ions which are less dissociable than the protein sulfhydryl-metal complexes. Stocken & Thompson (123) showed that BAL can reactivate brain pyruvate oxidase which has been inhibited by lewisite; Thompson & Whittaker (124) have demonstrated a similar reversal for brain pyruvate oxidase which has been inactivated by antimony, gold, or mercury. Barron and his co-workers (125) found, that of the trivalent arsenic compounds, lewisite had the greatest affinity for the sulfhydryl groups of the protein moiety of enzymes, that it inhibited all such sulfhydryl enzymes with the exception of D-amino acid oxidase, yeast carboxylase and transaminase and that BAL reversed the enzyme inhibition in every instance.

The capacity of BAL for mercaptide formation also has unfavorable, toxic effects in that it may combine with the metal ions of the metallo-protein enzyme systems. Webb & Van Heyningen (126) observed that phenol oxidase (Cu^{++}), carbonic anhydrase (Zn^{++}), catalase (Fe^{+++}) and peroxidase (Fe^{+++}) were inhibited markedly by BAL in concentrations ranging from 0.001 to 0.005 *M*; cytochrome oxidase was, however, not inhibited. Of a number of nonmetallo-protein enzymes which were tested, only glyoxalase, aldehyde mutase and phosphorylase were inhibited. Barron, Miller & Meyer (127) have pointed out that another factor, namely, the reducing power of BAL, may also be responsible for inhibition of enzyme activity. In contrast to the findings of Webb & Van Heyningen (126), they showed that cytochrome oxidase, succinic oxidase and, to a smaller extent, cholinesterase were inhibited by BAL. No correlation between enzyme inhibition and symptoms of BAL toxicity have yet been demonstrated in the whole organism (128, 129).

The administration of BAL causes a mobilization of metallic ions in cases of arsenic, cadmium or lead poisoning. Riker & Rosenfeld (130) found that the concentration of arsenic in the plasma of mapharsen poisoned cats showed a sharp rise immediately after the injection of BAL. Eagle, Magnuson & Fleischman (131) observed a marked increase in the urinary excretion of arsenic after BAL administration to arsenic poisoned rabbits. Increases in the urinary excretion of arsenic following BAL have also been obtained

in men with arsenical intoxication (132) and in normal individuals exposed to minimal concentrations of arsenical smoke (133). Tepperman (134) found that BAL and BAL-glucoside increased markedly the excretion of cadmium in rabbits poisoned by the intravenous injection of cadmium chloride. Ryder, Cholak & Kehoe (135) observed that, in cases of exposure of men to lead, the administration of BAL decreased the concentration of lead in the plasma and increased its excretion in the urine.

Because of the therapeutic importance of BAL, its metabolism is of special interest and may be considered at this point. Spray, Stocken & Thompson (136) have found that, after injection of BAL, the urine of rabbits shows a content of thiols, mostly dithiols, which is equivalent to about 20 per cent of the injected BAL. In BAL injected rats, there is a marked rise in the urinary excretion of neutral sulfur, inorganic sulfate and glucuronic acid, but no rise in ethereal sulfate (137). Peters and his co-workers (138) have shown, by employing S^{35} marked BAL, that most of the BAL sulfur is excreted in the neutral sulfur fraction.

Glucuronide and hippuric acid formation.—It is well known that many drugs and toxic substances are excreted in part as glucuronides. The combination of benzoic acid or its derivatives with glycine to form hippuric acids is of particular interest because it represents the *in vivo* synthesis of a $\cdot\text{CO}\cdot\text{NH}\cdot$ bond. Although little additional information has been gathered during the past few years on the mechanism of glucuronic acid formation, the development of Tollen's test into methods for the quantitative determination of glucuronides in urine and plasma promises to be of considerable aid in the study of this problem (139, 140, 141). Other hexuronic acids are also determined by this procedure.

Estimates of the normal excretion of glucuronic acid vary widely. Hanson, Mills & Williams (140) report an average daily excretion of about 1 gm. in man and about 140 mg. in the rabbit. The average values presented by Deichmann and Thomas (142) are much lower: 5.4 mg. in the rat, 35 mg. in the rabbit and 164 mg. in man. The excretion of glucuronide appears to be increased slightly but definitely in man on a protein diet and decreased in rats on a carbohydrate diet. Dziewiatkowski & Lewis (143) have observed that trimethylacetic (pivalic) and tertiary butylacetic acids are excreted as glucuronides; these are believed to be the first

reported instances of the conjugation of glucuronic acid with aliphatic fatty acids.

The role of the liver in glucuronide formation still appears to be controversial. Lipschutz & Bueding (144) had shown that glucuronides of *d*-borneol, *l*-menthol and avertin are formed actively by liver slices, only slightly by kidney slices, and negligibly by other tissues. Fishman's (73) observation of an increase of β -glucuronidase activity in the liver and kidney of animals fed borneol and camphor supports, although indirectly, the role of the liver in glucuronide formation. In apparent contradiction to these results are the observations of Deichmann, Kitzmiller & Witherup (145) to the effect that the conjugation of glucuronic acid with cyclohexanone is not decreased in rabbits with moderate or severe injury of the liver induced by phosphorus or by carbon tetrachloride-chloroform mixtures. However, it must be recognized that such damage may exist without interfering with the capacity of the liver to form glucuronides. Lipschutz & Bueding (144) had noted that fatty liver slices from guinea pigs poisoned with phosphorus continued to form glucuronides *in vitro*, although at a somewhat diminished rate, and that liver slices from guinea pigs poisoned with chloroform showed a highly active conjugation. Indeed, Snapper & Saltzman (146) have recently noted that in patients with liver disease glucuronide conjugation of benzoic acid persists when the conjugation of glycine has diminished. After a test dose of 5 gm. of benzoic acid, benzoyl glucuronide is excreted in patients with liver disease whereas it fails to be excreted in normal individuals; presumably, in the latter, the preferential pathway of conjugation with glycine is intact.

Borsook & Dubnoff (147) have revealed some of the mechanisms involved in the synthesis of hippuric acid from glycine and benzoic acid. By homogenizing liver with benzoic acid and glycine, they were able to get an appreciable yield of hippuric acid. The yield was doubled in the presence of adenylic acid and α -ketoglutaric acid. Presumably, the oxidation of the latter furnishes a supply of ATP which, in turn, provides the energy for the synthesis of the hippuric acid.

Metabolic adaptation.—It is well known that the administration of certain drugs may result in increased tolerance and may necessitate the use of higher doses to achieve the required therapeutic

effect. Evidence has recently accumulated to indicate that this effect is related to adaptations in the mechanisms involved in the metabolism of the drugs to which tolerance develops. The addition of various substrate to the media of certain bacteria and other microorganisms increases the activity of the enzymes acting on these substrates (148, 149). This phenomenon has been termed "enzyme adaptation," although Sevag (149) has protested the use of this term and has stressed the view that cells do not synthesize new "adaptive" enzymes but that changes in the metabolic environment increase to a manifest degree the activities of enzymes which are already present in the cells. A phenomenon similar to that existing in microorganisms has also been noted in mammals. Among the older observations are those of Weinland (150) who observed that invertase appeared in the blood after the intravenous injection of sucrose. Rats fed alcohol daily for a period of a month showed high normal values for alcohol dehydrogenase (151). Lightbody & Kleinman (152) found that the arginase activity of liver increased when rats were fed increased amounts of adequate protein.

As has already been noted, Fishman (72, 73) observed that the β -glucuronidase activity in the livers of rats increased after feeding of borneol and menthol; uterine β -glucuronidase activity increased after repeated injection of estrogenic substances. De Meio & Arnolt (28) reported marked increases in the capacity of liver and kidney slices for conjugating phenol after this substance had been fed to rats by stomach tube every two to three days for four to sixteen times. However, in a strain of rats in which the liver had only a slight or negligible capacity for conjugating phenol, the liver and kidney showed no increase in this capacity after repeated feeding of phenol (30). Gross & Thompson (153) found that dogs which had become tolerant to morphine excreted less morphine, 33 to 66 per cent of the administered doses, than did nontolerant animals in which the recoveries ranged from 80 to 92 per cent. Apparently in the tolerant animal, there was a greater capacity for destruction of the ingested morphine; this was also reflected in a smaller excretion of conjugated morphine. Tepperman & Bodansky (154) noted that when *p*-aminopropiophenone was injected twice daily for a period of two weeks into rats, the extent of methemoglobin formation in response to a dose of the drug at the end of this period was significantly less than in control animals. Since, in this instance,

methemoglobin formation is due to an unconjugated intermediate (154), the possibility exists that repeated administration increased either the activity of the enzyme conjugating mechanisms or of other enzyme systems concerned in the metabolism of the methemoglobin-forming intermediate.

The preceding observations show that the activity of enzyme systems which are concerned in the metabolism of drugs and toxic substances may be increased in response to repeated administration of small, nontoxic doses of these substances. The organism thus becomes capable of metabolizing these substances much more readily. The mechanisms by means of which the enzyme activity is increased appears worthy of further study.

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CLINICAL APPLICATIONS OF BIOCHEMISTRY¹

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The magnitude of the contributions of biochemistry to medicine, and conversely of the clinic to the biochemical laboratory scarcely requires comment. This review is intended to survey some of the collaborative fields in which activity has been productive or promising.

SERUM POTASSIUM

Methods.—Classical methods for determination of serum sodium and potassium remain time consuming and laborious—prohibitively so as concerns their effective use in routine clinical chemistry. Methods of requisite simplicity may well evolve by adaptation of the flame photometer (1) to biochemical estimations. In this instrument liquid samples containing alkaline earth ions are sprayed as a fine mist into a gas flame. Light from the flame is dispersed into a spectrum, and its intensity measured by photoelectric cell; after suitable amplification the resulting current actuates a null-point galvanometer which has been calibrated against standard solutions. Filters of maximum transmission appropriate to the wave lengths emitted by the activated ions permit estimations of sodium and potassium, for example, without prior separation, and in the presence of many extraneous materials normally present in native biological samples. Simple, rapid methods of preparing food, whole blood, serum, urine and feces for analysis have been described (2, 3, 4). Results obtained with experimental models developed by American Cyanamid Company and with early commercial models not specifically designed for use with biological materials are variable but encouraging (2, 3, 4). More recent commercial models² developed primarily for biochemical application are currently undergoing critical appraisal.³

Hald has modified (5) the dry ashing procedure for sodium and potassium in whole blood and serum. The iron phosphate precipi-

¹ The topics presented under this title are based on literature reviewed to November, 1947.

² Model 52-A, Perkin-Elmer Corporation.

³ Personal communication from Miss Pauline M. Hald.

tate is redissolved in concentrated hydrochloric acid, evaporated to dryness, and extracted with water, thus avoiding loss of potassium by occlusion.

Potassium intoxication.—Spontaneous and fatal potassium intoxication complicating familial nonhemolytic jaundice and chronic nephritis has been observed by Finch, Sawyer & Flynn (6). Association of characteristic electrocardiographic abnormalities and severe disturbance of myocardial function with serum potassium levels of 9.0 or more m. eq. per l. is now well established (6, 7, 8). Levels of 10.0 to 10.5 m. eq. per l. may be lethal in humans (6), although successful calcium therapy of complete heart block due to a level of 12.27 m. eq. per l. in a child has been reported (7). Transient therapeutic effects are obtained with sodium chloride (6) in chronic hyperkalemia. Combined sodium bicarbonate and sodium chloride have been effective in the treatment of acute potassium intoxication (4).

Diminished renal clearance of orally ingested potassium in chronic renal disease has been reinvestigated and confirmed by Keith & Osterberg (9). Mechanisms of the variable occurrence of high, low or normal serum potassium levels in uremia await explanation.

Demonstration of the grave prognostic significance of elevated serum potassium emphasizes that despite a voluminous literature, the possible role of potassium intoxication in shock remains but poorly assessed. In dogs in prolonged shock following muscle trauma, Holmes (10) has found elevations of less than 1.5 m. eq. per l. until about one and one-half hours before death. Serum potassium may then rise to an average of 9 m. eq. in about half the animals. These data support the general conclusion (10, 11) that hyperkalemia reflects a terminal loss of potassium from traumatized or anoxic tissues and is not directly concerned in the development or progression of the shock state in dogs. In the "crush syndrome" in humans, however, Bywaters (12) has observed serum potassium levels of 10.0 or more m. eq. per l. together with abnormalities of the electrocardiogram characteristic of potassium intoxication. Myocardial failure was considered the immediate cause of death in some of these patients.

Recent experiments of Moritz and co-workers (13, 14) are of absorbing interest. Pigs with extensive cutaneous burns rapidly (within a few minutes) develop serum potassium levels of 16 m. eq.

or more per l. These animals have abnormal electrocardiograms. Observations of the circulatory dynamics indicate that myocardial failure contributes importantly to death. Serum potassium increments in these pigs are derived in part from extravascular sources, but to a much greater extent from intact or lysed red blood cells. In dogs with similar cutaneous burns, in spite of the occurrence of massive hemolysis, potassium levels do not rise above 8.2 m. eq. per l. The normally different distribution of potassium and sodium between red blood cells and serum of the pig and dog is apparently concerned here. Potassium content of dog erythrocytes is low (9 mM per kg. of red cells) in contrast to that of pig (100 mM per kg.) and human (110 mM per kg.) erythrocytes (15). It is of interest that Ponder (16, 17) has observed for human red blood cells in a lytic system the loss of as much as 7 to 15 per cent of their potassium before any hemoglobin loss is detectable. The experiments of Moritz, Roos and their co-workers should prompt further evaluation, with due regard for species differences, of the importance of serum potassium elevation as a serious complication of prolonged shock.

Potassium deficiency.—Clinical occurrence of potassium deficiency has been reported in the sprue syndrome (18) and during the postacidotic stages of diabetic coma (19, 20) and infantile diarrhea (21, 22, 23). Outstanding studies by Darrow of the mechanisms of serum potassium depletion following acidosis or alkalosis (22, 24) emphasize the inadequacy of previous interpretations which assume intracellular and extracellular compartments locked against the passage of cations other than hydrogen and ammonium. Electrolyte balance studies indicate severe depletion of intracellular potassium in acidosis; its replacement by extracellular sodium may in fact produce the acidosis (22). Severe serum potassium deficits may then result (a) when loss occurs in the urine or stools and when in the postacidotic period; (b) when blood volume is restored; (c) when tissue cells rapidly regain potassium. Darrow has calculated that in infantile diarrhea losses of intracellular potassium may amount to more than one-fourth of the estimated potassium content of the baby. By adding potassium chloride to parenteral fluids and subsequently to the oral diet in the treatment of severe diarrheas, Govan & Darrow (23) achieved an astounding reduction of mortality—from 32 down to 6 per cent.

Observed electrolyte disturbances in diabetic acidosis are also

not satisfactorily explained by assuming shifts only of water and anions between intracellular and extracellular fluids, according to Danowski, Hald & Peters (20). Large exchanges of sodium and potassium between cells and extracellular fluids occur despite opposing concentration gradients of great magnitude. Exchange of these cations does not take place *in vitro* between an inorganic medium and red blood cells when conditions are such that metabolic activity is largely suspended (25).⁴ A most interesting and useful concept stems from these observations: transfer of sodium and potassium across cell surfaces in the intact animal is more closely associated with vital metabolic processes than with purely physical equilibrium forces (25).

PHOSPHATASES

Histochemical methods pertinent to the demonstration and study of phosphatases comprise a section of an excellent review by Dempsey & Wislocki (26). Recent studies more firmly establish the variations of serum "alkaline" and "acid" phosphatase activities encountered in physiological (27) and abnormal (28 to 33) clinical states. Technical modifications of methods, and lack of proportionality over wide ranges of units employed, invalidate quantitative comparisons of these reports. Possibility of dispelling the necessity for empirical interpretation of these data rests with investigations of the origins, specificities and functions of the phosphatases which are, therefore, the major concern of this survey.

Alkaline phosphatase.—It is reaffirmed that elevations in serum alkaline phosphatase activity are greater in obstructive than in nonobstructive jaundice (34). This observation has been the basis of studies of the origin of alkaline phosphatase as well as being of aid in differential diagnosis of jaundice, as discussed below. Tanturi and associates (35), while confirming this relationship, are unable to differentiate the relative roles of decreased biliary excretion of phosphatase as opposed to increased phosphatase production in augmenting the serum phosphatase activity of dogs subjected to bile duct ligation or chloroform liver injury. Wachstein & Zak (36) note histochemical evidence of increased phosphatase activity not

⁴ The seeming discrepancy between this observation and those of Ponder (16, 17), showing loss of potassium from red cells in a lytic system, is probably adequately explained by important differences in the suspending media employed.

only in dilated bile capillaries, but also in adjacent liver cells in dogs with obstructive jaundice. In human necropsy material, increased alkaline phosphatase activity occurs in actively regenerating areas of nonobstructive liver lesions and in obstructive cases is conspicuous in dilated bile capillaries (36). The difficulty of evaluating the true extent of cellular damage accompanying obstruction, and of intracanalicular and capillary stasis in primary parenchymal injury, is recognized by the authors. Evidence of hepatic production of alkaline phosphatase following 70 per cent partial hepatectomy in rats is reported by Oppenheimer & Flock (37). Increases in serum alkaline phosphatase activity closely parallel increases in liver phosphatase and in mitotic activity. In histochemical and chemical studies, Vail & Kochakian (38) have demonstrated large increases in liver phosphatase during the administration of adrenal cortical extract to rats. Increases in phosphatase are more rapid than increases of glycogen in these livers. Dalgaard (39) has noted increased alkaline phosphatase activity in the duodenum of rats following bile duct ligation. Gad (40) found in dogs greater absolute elevations of intestinal (jejunal) than of hepatic phosphatase four to five days after ligation of bile ducts. Hoffmeyer and associates (41), however, demonstrated no increase in liver or intestinal phosphatase seventeen to twenty hours after similar operations in rabbits.

The alkaline phosphatase responsible for increased serum activity in patients with hepatic damage has been differentiated by the effect of cyanide ion from the enzyme normally present in serum by Drill & Riggs (42). In normal sera, 0.0001 to 0.1 *M* sodium cyanide has little effect on phosphatase activity. In the sera of patients with hepatic damage, 0.01 *M* sodium cyanide decreased initially high phosphatase activities an average of 9.7 units, and increasing cyanide concentration reduced the activity to normal levels, but not below. A report by Roche (43) that elevation of serum alkaline phosphatase activity in a variety of pathological states is due to a dialyzable material which activates liver as well as serum phosphatase apparently remains unconfirmed.

The phosphoesterase preparation from calf intestinal mucosa has been extensively studied by Zittle who has recently summarized his work (44) as well as that of Winnick (45, 46) and Schmidt & Thannhauser (47). This preparation hydrolyzes in alkaline media both diesters (nucleic acid to mononucleotides) and mono-

esters (mononucleotides to nucleosides and phosphoric acid). A single enzyme probably effects both of these hydrolyses, as well as the hydrolysis of the synthetic phosphoamides prepared by Winnick (46), and the hydrolysis of phosphocreatine (45). This enzyme, the activity of which appears to be chiefly concerned with the disposal of food materials, is probably identical with the alkaline phosphatase of the blood. It is possible to distinguish ribonuclease from the intestinal phosphodiesterase by the greater lability of the latter to heat (47) and by the greater concentration of ribonucleic acid necessary for optimum activity of ribonuclease (48).

An interesting clinical experiment making use of theoretical knowledge of phosphatase mechanisms has been reported by Black & Kleiner (49). These authors attempted to inhibit high energy phosphate bond reactions in human malignancies by the administration of sodium fluoride, iodoacetic and malonic acids. Temporary hematologic remissions were noted in leukemias. In a variety of other malignancies shrinkage of tumor mass, relief of pain, increase of body weight and well-being and definite retrogressive changes on biopsy were found. Adaptation to one after another of the inhibiting agents by the development of accessory pathways of phosphate bond formation was considered to be responsible for the temporary nature of remissions. Adaptation to all three of the inhibitors used appeared to these investigators to be the limiting factor in continued therapeutic effect.

Atkinson & Elftman have described cyclic alterations in alkaline phosphatase activity in human endometrial biopsies (50). Activity is increased during the proliferative phase, falling sharply prior to menstruation.

Acid phosphatase.—Serum acid phosphatase activity in the diagnosis of carcinoma of the prostate has been discussed by Woodard & Dean (32). Herbert has summarized results of several groups of investigators (51). Elevated serum acid phosphatase values were found in 58 per cent of 455 cases of prostatic carcinoma without metastases and in 86 per cent of 285 cases with metastases. Determination of acid phosphatase activity of biopsy specimens has been used by Dean & Woodard (52) in the differential diagnosis of tumors of the prostate and adjoining areas of the rectum and bladder. Using similar methods in the evaluation of the response of prostatic cancer to hormone therapy, these authors find that

estrogen-induced reductions of phosphatase activity may persist in spite of resumption of active tumor growth in cases which relapse.

Histochemical studies of acid phosphatase activity have been correlated with histologic regressive changes in serial biopsies during estrogen therapy of prostatic malignancies by Fergusson (33). In lymph nodes involved by metastases, increased phosphatase activity is found in occasional instances other than primary carcinoma of the prostate. There is inconclusive evidence of greater phosphatase activity in the posterior portions of normal prostate glands, the area in which malignancy of the prostate most frequently arises.

Specific inhibition of the serum acid phosphatase of prostatic origin by incubation of serum at 37°C. at pH 7.4, or by the addition of two-fifths volumes of ethyl alcohol to serum, has been reported by Herbert (51). The procedure is successfully used in distinguishing equivocal elevations of serum phosphatase activity accompanying prostatic carcinoma from those resulting from other causes. Abul-Fadl & King (53) have noted similar inhibition of acid phosphatase activities of bile, kidneys, red cells and prostate by alcohol; activities of adrenal, intestine, liver, pancreas, spleen and thyroid are unaffected. These same authors report specific inhibition of red blood cell phosphatase activity due to hemolysis by addition of 0.1 ml. of 20 per cent formaldehyde to buffer-substrate-serum mixtures prior to incubation.

Estimation of urinary phosphatases by the use of monophenyl phosphate as substrate and a diazo reagent for colorimetric estimation of liberated phenol is described by Burgen (54). Acid phosphatase excretion in women averages 50 to 60 King-Armstrong units in twenty-four hours. In males similar values in the first decade rise to a maximum of 250 to 400 units in the fourth, fifth and sixth decades, then fall abruptly to about the female level. Normally high excretory levels in men with suprapubic cystostomies suggest that the urinary activities arise by renal excretion, rather than by admixture of prostatic phosphatase during voiding. No significant alterations of phosphatase excretion are reported in benign prostatic hyperplasia, carcinoma of the prostate, or glomerulonephritis. Delory (56) found no correlation between acid phosphatase activity of the semen and the number or motility of sperm in sterility.

In sperm a phosphatase capable of hydrolyzing adenosinetriphosphate to adenylic acid and thus distinct from the acid phosphatase of seminal fluid has been demonstrated by MacLeod & Summerson (55). Its function is not clear. The reaction is not accompanied by phosphate transfer to any of a number of potential acceptors studied, nor does the energy yielded increase or prolong the motility of sperm in the absence of glucose *in vitro*. The reaction does not, furthermore, modify the normal anaerobic catabolism of sperm as measured by lactic acid production in the presence of glucose.

BLOOD IODINE

Thyroid and thyrotropic hormone and antithyroid substances have been considered in a recent review by Lukens (57).

Full clinical use of blood iodine determinations in the diagnosis and therapeutic control of abnormal thyroid states has awaited the following developments: (a) clarification of the relationships between protein-bound, "precipitable" or "hormonal" iodine and inorganic iodine of the serum, analysis of their significance in terms of thyroid activity, and precise definition of normal and abnormal values; (b) reduction of volume of sample required; (c) description of rapid, effective, and reproducible methods for the separation of protein-bound from inorganic iodine; (d) simplification of procedures for the quantitative collection of small amounts of iodine after oxidative destruction of large amounts of organic material; (e) standardization of colorimetric, volumetric or titrimetric methods for iodine estimation. Some of these requirements have been achieved.

(a) Normal presence of only small amounts of iodine in red blood cells and inorganic serum fractions has been confirmed (58). Wide variation during and following iodine administration has been demonstrated (59).

The most conclusive evidence thus far obtained for the actual identity of serum-protein-bound iodine with circulating thyroid hormone has recently been reported by Taurog & Chaikoff (60). On the bases of solubility, optimum conditions for crystallization, and distribution between immiscible solvents, 90 per cent of the protein-bound I^{131} in the plasma of rats fed the isotope could not be distinguished from thyroxine.

Studies correlating whole blood iodine levels with clinical manifestations and basal metabolic rate in thyroid and nonthyroid disease have been extended by Curtis & Fertman (61). A mathematical relationship developed by Lowenstein and colleagues between protein-bound serum iodine and basal heat production in untreated and iodine-treated hyperthyroidism (62) and in treatment with thiouracil (63) is of theoretical interest.

Protein-bound serum iodine levels have been found an extremely sensitive index of a wide range of thyroid activity in extensive studies by Winkler, Riggs, Man, Danowski and their collaborators. In myxedema, response of plasma iodine to dessicated thyroid extract (64) is in marked contrast to the tolerance for this material displayed by euthyroid individuals (65). As an assay of the severity of hyperthyroidism and its response to thyroidectomy (66) or in the establishment of optimal dosage of iodine and anti-thyroid substances (67, 68), plasma iodine levels prove more precise than do clinical appraisal, basal metabolic rate, or serum cholesterol levels (66). Man and co-workers suggest that the demonstration of serum precipitable iodine levels of less than 2 $\mu\text{g.}$ per cent may be a valuable aid in the early diagnosis of cretinism (69). Recognition of the condition by other criteria is not usually possible prior to the appearance of irrevocable changes.

Using radioactive iodine, Taurog & Chaikoff (70) have found the turnover rate of protein-bound serum iodine in 8 to 10 kg. dogs to be 50 to 100 $\mu\text{g.}$ in twenty-four hours. This corresponds to the turnover every four to seven and one-half hours of the amount normally present in the circulation. In rats, a significant decrease in protein-bound serum iodine is apparent as early as four hours after thyroidectomy; and twelve-hour levels decline to 30 per cent or less of normal (71). In rats and guinea pigs given thyrotropic hormone, serum iodine levels may rise from 2.2 to 5.8 $\mu\text{g.}$ per cent in four days (71).

Essential agreement on normal values for protein-bound serum iodine has been reported by various workers. Winkler, Riggs & Man (64, 65) find extreme normal values of 3.0 to 9.0 $\mu\text{g.}$ per cent. Talbot and his co-workers (72) report values of 6.0 to 8.4 $\mu\text{g.}$ per cent for normal adults, 4.0 to 7.0 $\mu\text{g.}$ per cent for children. Bassett, Coons & Salter define normal levels of 4.0 to 9.0 $\mu\text{g.}$ per cent (73).

(b) Reduction in size of samples required for blood iodine estimation has been accomplished by adoption of micro and semi-micro methods. Sappington, Halperin & Salter (74) and Salter & McKay (75) have obtained analyses satisfactory for clinical purposes using 1 cc. of serum. Similar accuracy has been achieved with methods using 3, 4, or 6 cc. samples (58, 68, 71, 72, 76).

(c) Methods applicable to the separation of protein-bound from inorganic iodine of serum have been reviewed by Wilmanns (77). Precipitation with heat and acetic acid has been shown to yield satisfactory results except in the presence of elevated inorganic iodine levels, which produce spuriously high values for protein-bound iodine (73, 78). Taurog & Chaikoff (76) find that the precipitate formed by zinc sulfate and sodium hydroxide retains a small, variable amount of inorganic iodine despite three washings with distilled water.

(d) Destruction of organic materials in iodine-containing samples continues to be accomplished by acid-permanganate ashing by Riggs, Man and associates (58, 66, 79). This procedure has been adapted to subsequent distillation in the Chaney still (80) by Talbot and co-workers (72). Taurog & Chaikoff have studied the reactions involved (76) and have utilized this distillation procedure following digestion with chromic and sulfuric acid for subsequent studies of thyroid metabolism (71, 81). They point out theoretical advantages of wet ashing procedures over the dry ashing with sodium carbonate used by Salter and his co-workers (74, 75). Spector & Hamilton (82) have studied the combustion of iodine-containing organic materials in the Parr oxygen bomb, but did not apply the method to blood protein precipitates.

(e) Determination of iodine following oxidation has been accomplished by titration of iodine (62, 63) or of iodate (58, 72) with starch in the presence of iodide, or by colorimetric assay of the catalytic effect of iodine on the reduction of ceric sulfate by arsenious acid (74, 75, 76, 80). The latter avoids the uncertain color reactions associated with varying starch samples (83), but requires painstaking exclusion of extraneous materials from the reaction (74, 75, 76).

The foregoing summary indicates that a firm basis has been established for the use of blood iodine determinations in the study of thyroid disease. Considerable progress has been made toward the goal of routine clinical practicability of methods.

ELECTROPHORESIS

Numerous reports of the application of electrophoresis in the analysis and separation of protein materials have continued to appear. For excellent recent accounts of the use of the technique in biological and medical studies, the reader is referred to the review of Leutscher (84) and of Stern & Reiner (85).

LIVER FUNCTION STUDIES

Current concepts of jaundice have been well presented by Young (86). General reviews of liver physiology (87) and liver function tests (88) have appeared. Search continues for a test to supersede all or most others. The multiplicity of the functions of the liver and its well known reserve render attainment of this objective unlikely. Use of carefully selected groups of laboratory aids is therefore a continuing necessity (89, 90). The following paragraphs summarize certain newer procedures and newer interpretations of old ones.

Bilirubin.—The level of the total serum bilirubin in health as determined by the Malloy & Evelyn method (91) is below 1 mg. per 100 cc. (92, 93). Slight elevations may denote mild liver disease (92). Greater elevations, while obviously indicating disease, very often do not accurately define it.

The significance of partition of the total serum bilirubin into direct and indirect fractions according to its reaction with Ehrlich's diazo reagent in the absence or presence of alcoholic substances is still under study thirty years after its description by van den Bergh. Some clinical but scant basic progress has been made. Gray (94) has found the direct/indirect quotient measured by the Malloy-Evelyn method (91) of some reliable diagnostic value in jaundice. Quotients below 40 were found only in patients with hemolytic icterus. Sixty cases of obstructive and parenchymal jaundice all had quotients above 40 as did also some cases of hemolytic jaundice. The measurement will not distinguish obstructive from parenchymal jaundice.

Direct reacting bilirubin is still believed by many students of the problem, as postulated originally by van den Bergh, to be formed from indirect reacting bilirubin by passage through the liver cell. Watson (93) reviews and subscribes to the concept of a chemical union involving valence bonds between bilirubin and

globin to form the indirect fraction, the direct fraction being a more loosely bound sodium bilirubinate-protein complex. Gardikas, Kench & Wilkinson (95) state that they find the direct and indirect reactions most likely to be based upon differences in the pigments themselves but present no supporting data. As still a third explanation Gray & Whidborne (96) revive an earlier theory that a catalytic factor appears in the serum in regurgitation jaundice and facilitates the diazo reaction. It is stated (95) that search has failed to reveal either an accelerating factor in direct reacting or an inhibiting agent in indirect reacting serum. More and better data are needed.

In an extensive study Gray & Whidborne (96) were impressed by the occasional similarity of the rate of the van den Bergh reaction in hemolytic icterus to that in regurgitation jaundice. Curves were plotted of the rate of azobilirubin formation from either direct or indirect reacting fractions as percentage of total bilirubin. The regurgitation jaundice type of curve when found in hemolytic icterus was frequently associated with evidence of impairment of hepatic excretory mechanism as manifested by increased urinary urobilinogen and urobilin. A degree of regurgitation type of liver injury as a secondary complication of some cases of hemolytic jaundice is generally recognized. It can account for the variable curves of Gray & Whidborne whether it provides a more loosely bound pigment, a different pigment, or a factor accelerating the Ehrlich reaction.

The separation of direct reacting bilirubin into prompt (1 min.) and delayed (next 14 min.) components by Ducci & Watson (97) is found by Gray & Whidborne (98, 94) to have no clinical significance and to represent merely a failure of strong bilirubin solutions to conform to Beer's law by absorbing light in direct proportion to their concentrations. Gray & Whidborne (98) believe their evidence weakens the possibility (97) that delayed direct bilirubin can be the same as the indirect reacting variety.

Of the methods for determining serum bilirubin that of Malloy & Evelyn (91) appears to have the widest acceptance, and most simplicity and accuracy (96, 97). The use of pyridine in low concentration in place of alcohol in the indirect van den Bergh reaction is said to offer many advantages (95). It should also be mentioned that the serum bilirubin loading test of excretory ability

which heretofore has been regarded as highly sensitive and reliable was found by Neeffe (99) to give very inconsistent results.

Biliverdin determination in the serum is of interest. A spectrophotometric method, which is simple if interfering blood pigments and turbidity can be excluded, has been described by Larson, Evans & Watson (100). Biliverdin is considered an intermediate in the formation of bilirubin from hemoglobin. None is found in nonjaundiced persons nor in cases of hemolytic icterus even with total bilirubin levels as high as 14 and 30 mg. per cent. In patients with obstructive or parenchymal jaundice the serum biliverdin level is significantly elevated, as high as 2.2 mg. per cent. Evidence is presented that biliverdinemia is abolished by improvement in the nutrition of the icteric patient.

Bilirubinuria may appear early in liver disease before appreciable elevation of serum bilirubin is detected (101). A very simple, reasonably quantitative and highly sensitive and specific method for its detection has been developed from the Harrison spot test by Watson & Hawkinson (102). The method is superior to older ones as well as to the improved methylene blue test (103, 104).

Urobilinogen.—The term urobilinogen is used by Watson (93) to include all the fecal and urinary chromogens of the urobilin type. The estimation of urobilinogen by the clinically feasible method of Watson *et al.* (105) also includes urobilin, stercobilin and the related pigments which are reducible with ferrous hydroxide to Ehrlich reacting chromogens. It includes as well some incompletely defined substances which are probably not urobilinogens (106). These substances, however, are not extractable by petroleum ether and are, therefore, not included in the more laborious but more quantitative method for urobilinogen of Schwartz, Sborov & Watson (106). Sources of high values (107) and correction of the original (105) calibration of the Evelyn colorimeter with the pontacyl dye standard are given by Watson & Hawkinson (108).

Greater recognition of the diagnostic value of urobilinogen determination in hemolytic and hepatic disease has occurred (86, 107, 108). The simplified semiquantitative procedure and the feasibility of using random fecal samples and two-hour urine samples, with precautions (108), will extend its use. High fecal values are believed to be indicative with rare exceptions of in-

creased blood destruction. Values less than 5 Ehrlich units (105) found in four daily random samples are generally equivalent to finding less than 5 mg. urobilinogen per day in the more arduous method with complete four-day collection (108), and are similarly indicative of complete biliary obstruction.

Urine urobilinogen values fluctuate considerably during the twenty-four hours with the 2:00 to 4:00 P.M. collection most likely to be highest (105). The normal range is 0.6 to 0.8 Ehrlich units per 100 cc. urine by the simplified method (105). Values above two units are distinctly abnormal (109). Ehrlich units are only very roughly proportional to urobilinogen as measured in milligrams by the more exact method (106), being greater by inconstant factors roughly from 1.1 to 1.5 and occasionally to several times as much particularly in the low ranges.

A urobilinogen tolerance test of liver function gave too variable results when applied to the study of liver involvement in icterus neonatorum (110).

Bromsulfalein excretion.—Long regarded as probably the simplest of the dye excretion tests though still not informative enough for wide usage the bromsulfalein test has been improved until it is the most sensitive indicator of liver dysfunction in early hepatitis (111). The improvement has resulted from (a) increasing the load on the dye excretion mechanism of the liver from 2 mg. of dye per kg. body weight to 5 mg. per kg. (112); (b) better standardization of the normal excretion curve (113); and (c) adaptation of the procedure to the photoelectric colorimeter (114).

Ingelfinger (115) and associates have made interesting use of bromsulfalein in studying human hepatic blood flow. They found that change from supine to upright position occasioned a sharp decline in dye removal by the liver as detected by hepatic vein catheterization. Bromsulfalein removal during constant intravenous injection after an initial storage-saturating dose approximates 3 mg. per min. per sq. m. body surface. The normal removal constant for the dye is 10 to 15 per cent per min. of the amount present in the blood stream. During cardiac failure this drops to 6 to 8 per cent and in cirrhosis often to 1 to 4 per cent.

Serum protein flocculation and turbidity tests.—These empirical tests have wide clinical appeal and usefulness because of their simplicity. In general their biochemical basis remains unexplained. The cephalin cholesterol flocculation test of Hanger (116) is the

most sensitive simple screening test for early acute infectious hepatitis (111, 113). The thymol turbidity test of Maclagan (117) is a sensitive indicator of persistent hepatitis. In uncomplicated cases it may remain positive twenty to thirty days beyond the time at which the cephalin cholesterol flocculation has returned to normal (111, 118, 119). The thymol flocculation test of Neeffe (118) is even more sensitive to liver impairment in late hepatitis than is the thymol turbidity. It is performed by merely reading the degree of flocculation in the thymol turbidity tubes approximately eighteen hours after the thymol turbidity test has been read. Several other similar tests have been described—the colloidal gold (120, 121), the colloidal scarlet red (122, 123), the cadmium turbidity (124).

The mechanism of these tests when better understood promises to be both interesting and important evidence concerning the functions and possibly the origin of the serum proteins. In the absence of serum albumin electrophoretically pure γ -globulin flocculates with standard cephalin cholesterol reagent (125, 126). Moore and co-workers (125) have found that the minimum quantity of γ -globulin necessary to produce flocculation lies between 0.22 and 0.11 mg. No difference in sensitivity was detected between γ -globulins from normal or from hepatitis sera (125). In a preliminary report Maclagan & Bunn (127) have found the normal less sensitive (0.5 mg.) and hepatitis γ -globulin more sensitive (0.05 mg.). A mixture of hepatitis α - and β -globulin was also sensitive (0.06 mg.), but a mixture of normal α - and β -globulins was not. Inhibition of flocculation occurred when 5.9 mg. normal albumin was added in the reaction containing 0.33 mg. γ -globulin, but a similar quantity of hepatitis albumin merely reduced the flocculation from 4+ to 3+ (125). Maclagan & Bunn (127) found 100 parts normal albumin were required to inhibit one of γ -globulin. In this delicate reaction minor differences in the conditions may be responsible for divergence of results. It is of interest that γ -globulin separated at pH 8.5 in barbital buffer did not flocculate with the cephalin cholesterol reagent as did that obtained at pH 7.4 with phosphate buffer (125).

Stabilization of the cephalin cholesterol reagent has presented difficulties. Undesirable potentiation of sensitivity by exposure of the reagent to light has been generally avoided since its recognition (128). A more light stable reagent, also more suitable for testing serial dilutions of the serum, has been obtained (129) merely by

adding the cephalin cholesterol antigen to water at room temperature instead of at 70°C. as originally (116) described.

Alteration in the apparent proportions of the components of the serum proteins by simple dilution before measurement electrophoretically or by salt precipitation is well known. Makari (130, 131) has reported that increasing the dilution of normal serum beyond that customary in the cephalin cholesterol flocculation discloses a zone of flocculation. Flocculation increases from zero at the standard dilution of 1:20 to a peak of 3 to 4+ at 1:400 with decline to zero again upon dilution beyond about 1:6000. Makari postulates "an inhibiting factor" to be present in the serum which is diluted out earlier than "the flocculating factor" (130). These are not defined as albumin and γ -globulin but Makari states the inhibiting factor is present in greatest concentration in an albumin residue after salt precipitation. A binding of γ -globulin by albumin to prevent flocculation as supported by Wunderly & Wuhrmann (132) would be a possible explanation if this reaction could be shown to be inhibited by the dilutions used. Further data are needed. Whatever the factors are which may be essential to a positive flocculation in pathologic sera, other than the appropriate γ -globulin to albumin ratio, they are also present in the sera of premature infants during the first days of life (133) and of normal dogs and rabbits (116).

The thymol turbidity reaction has appeared to involve a mechanism fundamentally different from cephalin cholesterol flocculation according to clinical (118, 135) and laboratory (136) observations. MacLagan (117) and Recant, Chargaff & Hanger (136) have implicated a lipid-containing fraction of the serum in the thymol reaction. Cohen & Thompson (134) have found that the thymol reaction precipitates primarily β -globulin from either normal or hepatitis serum. The major change in electrophoretic pattern of hepatitis serum following thymol treatment is a 50 per cent reduction in β -globulin as against an average decrease in γ -globulin of about 4 per cent (134). Although thymol reduction of β -globulin is much smaller in normal serum the precipitate is indistinguishable electrophoretically or by the ultracentrifuge from that of the pathologic sera. The beta anomaly always disappears after thymol. Cohen & Thompson point out that the derangement of serum lipid in hepatic disease may be qualitative rather than quantitative (137) despite the lack of evidence for an abnormal lipid (136). The

critical alteration may be nonlipid, since ether-extracted serum may be resensitized to thymol by addition of lipid only if it was thymol turbidity positive before extraction (136). Contrary to the observations of Recant, Chargaff & Hanger (136), Maclagan & Bunn (127) report faint turbidity to thymol of pure γ -globulin and much increase in turbidity upon addition of cephalin or lecithin. They find turbidity abolished by normal, but not by hepatitis albumin.

Unlike the thymol reaction but similar to the cephalin cholesterol flocculation the colloidal gold reaction with serum depends upon γ -globulin and is inhibited by albumin (120, 126, 127). Alpha and β -globulin mixtures are reported to be even more inhibitory than albumin (127).

The cadmium turbidity test described by Wunderly & Wuhrmann (124) has technical advantages of simplicity of reagent (0.4 per cent $\text{CdSO}_4 \cdot 8 \text{H}_2\text{O}$) and speed of performance (read 5 min. after adding 0.2 cc. reagent to 0.4 cc. serum), but is somewhat less sensitive (132). Its turbidity-inducing property is said (132) to depend upon dehydration of protein molecules and it is inhibited by albumin of lower concentration than is either the cephalin cholesterol or thymol turbidity test. The test should be given a more thorough trial than has been reported.

Normal values for the flocculation tests have been well studied (111, 113, 138, 139). Advantages of measuring turbidity by other than visual standards (117) has stimulated development of spectrophotometric (140) and photoelectric (139) calibrations for turbidity readings, in addition to a photoelectric turbidimeter (141). This latter instrument possesses definite advantages since it responds to the scattering of light relatively independently of color.

The clinical distinction between hemolytic and nonhemolytic icterus generally presents less difficulty than the differentiation of obstructive from nonobstructive jaundice. One of the latest reported attempts to make the latter differentiation is Maclagan's interpretation (138) of the values of serum alkaline phosphatase and thymol flocculation tests in two hundred consecutive cases of jaundice. All cases with serum alkaline phosphatase levels greater than forty-two King-Armstrong units were obstructive; all with levels greater than thirty-five King-Armstrong units together with negative or weak flocculation were likewise obstructive. All with phosphatase less than fifteen units or with flocculations of 2+ or

more were nonobstructive. These criteria gave 79 per cent positive and correct diagnoses. It is to be expected that exceptions to these limits may be found. Phosphatase levels in nonobstructive jaundice above the limit of forty-two have been reported in Bodansky units (142) and MacLagan himself (143) has noted values above forty-two in subacute hepatitis and cirrhosis. If these or similar exceptions have elevated thymol flocculations the criteria may still be precise.

No one of the flocculation tests has all the properties of any other from the clinical standpoint (111, 117, 138). These differences probably rest in as yet unrecognized normal and abnormal components of the serum (127, 130, 134, 136, 144). The serum protein tests for disease reviewed here represent only recent developments in an already partially explored field (145).

A valuable guide in the assessment of chemical data in relation to the liver is afforded by histologic examination of liver biopsy specimens. A new modification of the biopsy needle with references leading to earlier types deserves listing (146). Critical correlations between laboratory studies and biopsy findings have been made (89, 147, 148, 149).

PORPHYRINS

Porphyrin analysis is still rather difficult for the routine laboratory, interesting and clinically important (150) though such data could prove to be. An excellent review of present knowledge of urinary coproporphyrins by Watson & Larson (151) is recommended.

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BIOCHEMISTRY OF THE HORMONES

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THYROID

In outline the function of the thyroid gland in the elaboration of its internal secretion may be summarized as follows: The gland has the capacity of removing circulating iodide which is in turn combined with an organic molecule, very probably tyrosine. Two molecules of di-iodotyrosine are oxidatively coupled with loss of a side-chain to form a molecule of thyroxine. Thyroxine is stored in the gland in the form of thyroglobulin and is secreted into the blood stream under the influence of the thyrotropic hormone as a lower molecular weight protein or protein-split product. Concomitantly there is hypertrophy of the acinar cells, an increase in thyroid weight, and an increased capacity of the thyroid to accumulate iodine. The mechanism of action of the thyroid hormone on the peripheral tissues is still obscure.

Vanderlaan & Vanderlaan (1) have shown that thyroid depleted of hormonal iodine is still capable of concentrating large quantities of iodine from injected potassium iodide. A gradient exists between serum and thyroid iodide and is increased tenfold with thyroid hyperplasia induced by propyl thiouracil. As a result of hypertrophy and hyperplasia, the thyroid may accumulate thirty or more times the amount of iodide concentrated by a normal gland in equilibrium with similar serum iodide concentration. This preferential concentration of iodide by the thyroid tissue is inhibited by thiocyanate but not by sulfaguanidine. Taurog *et al.* (2) have similarly concluded that in the goitrous gland and probably in the normal gland a mechanism exists for fixation of iodide which is independent of the subsequent conversion to di-iodotyrosine and thyroxine.

Wolff *et al.* (3) have shown that potassium thiocyanate inhibits the accumulation of iodine by thyroid *in vitro* and *in vivo* provided the concentration remains high. Under these conditions the compound also depresses di-iodotyrosine and thyroxine synthesis. Stan-

¹ This review covers the period from November, 1946 to December, 1947.

ley & Astwood (4) have assayed in man a number of antithyroid compounds, the primary action of which is to prevent the synthesis of thyroxine. The hormone stored in the gland continues to be secreted and, consequently, the thyroid iodine decreases rapidly. After the administration of iodine¹³¹ and while the gland is rapidly accumulating iodide, the goiterogen is administered and the varying degrees of inhibition are classified. 2-Mercaptoimidazole showed ten times the activity of thiouracil and was the most active of the compounds investigated. Von Berde (5, 6) reported that thiouracil inhibited the secretion of "thermothyryn A" as well as thyroxine; and since the former substance according to Mansfeld (7) does not contain iodine, he concluded that compounds of the type of thiouracil do not act exclusively by inhibition of an iodination reaction. Cullumbine & Simpson (8) have found that thiothymine (2-thio-5-methyl uracil) possesses both antithyroid and hemopoietic properties. Mackenzie (9) from a study of the effects of iodide administration on the action of different goiterogenic drugs in the rat has concluded that a different reaction is affected by sulfonamides than by thiouracil, thiourea and *p*-aminobenzoic acid.

In continuation of earlier investigations from their laboratory Chaikoff, Taurog & Reinhardt (10) have found that following thyroidectomy there is a noticeable decrease in the protein-bound iodine of rat plasma. In contrast to the normal animal where 90 per cent of the injected radio-iodine after twenty-four hours was protein-bound, the completely thyroidectomized rat showed only 5 to 10 per cent of the plasma radio-iodine in combination with protein. Thyrotropic hormone increased the level of plasma protein-bound iodine in rats and guinea pigs. The incorporation of inorganic iodine into protein-bound iodine of the plasma may therefore be considered an index of thyroid activity. Under the assumption that the turnover rate of protein-bound iodine of plasma is very nearly equal to the rate of secretion of hormonal iodine by the gland, Taurog *et al.* (11) concluded that in dogs the turnover was from 50 to 100 μ g. per twenty-four hours, and the formation of an amount of protein-bound iodine equal to that contained in the whole circulating volume occurred every four to seven and one-half hours. Other figures for the secretion of iodine or thyroxine by the thyroid given by different procedures are listed in this paper. Hurst & Turner (12) have also compiled values for

thyroid secretion by various species of animals. Taurog & Chaikoff (13) from specific activity time curves, following a single intravenous injection of radio-iodine, concluded that the average rate of thyroxine secretion was 2 μ g. per 100 gm. of body weight per twenty-four hours or that the thyroid gland in twenty-four hours secretes an amount of thyroxine equal to that present in the gland. From a consideration of these data, the authors have concluded that diiodotyrosine fulfills three necessary conditions as the precursor of thyroxine. Parkes (14) tested the biological activity of thyroid glands from five different species and compared this with the total iodine and acid-insoluble iodine fractions of the preparations. There was no evidence of an inactive product containing acid-insoluble iodine. The acid-insoluble iodine, therefore, of thyroid preparations unlike artificially iodinated protein (15) is all thyroxine iodine.

Junqueira (16) has shown that fragments of the thyroid glands of young white rats maintained *in vitro* for ninety-six hours were capable of responding to thyrotropic hormone stimulation by an increase in the amount of intracellular colloid. This took place within 30 minutes of the time of application and secretion droplets and the release of intrafollicular colloid were observed. Sodium iodide inhibited the action of thyrotropic hormone on the secretion and release of colloid. Dvoskin (17) has described an assay technique for the thyrotropic hormone based on the formation of intracellular colloid droplets which is considerably more sensitive than increase in cell height or glandular weight. Albert *et al.* (18) found that the addition of elemental iodine to a pituitary extract resulted in abolition of 90 to 100 per cent of the thyrotropic activity without causing any change in the gonadotropic action of this product, and the inactivation was proportional to the amount of iodine. The failure of the iodine-treated pituitary extract to exert its thyrotropic activity could not be ascribed to the biological action of iodine, to the action of any thyroxine that might have been formed in the reaction, nor to an effect on tissue absorption. The same authors (19) showed that the thyrotropic activity of this iodinated material could be restored by treatment *in vitro* with various goiterogenic and nongoiterogenic substances, all of which were reducing agents, and (20) that various goiterogens augmented the response to thyrotropin even though the agent was removed prior to bioassay. Chicks receiving a standard amount of

thyrotropic hormone showed a marked augmentation in response when thiouracil or 3-phenyl amino-methyl-2-mercaptylthiazoline, a goiterogen of similar potency, was administered simultaneously in the diet (21).

The effect of thyroid hormone, or the lack of it, on various metabolic processes in the body has been investigated. Mayer (22) concluded in agreement with the literature that beagles and probably all puppies do not need a functioning thyroid under ordinary conditions. Drill & Truant (23) found that the thyroid hormone influences the reactions involved in the conversion of carotene to Vitamin A, since normal rats could be maintained with supplements of either Vitamin A or carotene, whereas thyroidectomized rats could not be maintained with carotene, exhibiting ocular changes and weight loss, as well as increased mortality. Tissieres (24) reported a decrease in the cytochrome-*c* of the hind leg muscles of rats following either thyroidectomy or treatment with methyl thiouracil while thyroxine increased the cytochrome-*c* content of either normal or thyroidectomized rats. Steiner & Kendall (25) report that dogs fed thiouracil, together with cholesterol in cottonseed oil, exhibited arterial lesions after a year similar in distribution and character to those seen in human atherosclerosis and arteriosclerosis.

Taurog & Chaikoff (26) have presented convincing evidence that the circulating hormone is thyroxine. Harington (27) has reviewed this subject and arrived at a similar conclusion. Gross & Leblond (28) have studied the distribution of I^{131} and thyroxine in tissues, fluids and excreta after administration of large doses of labeled thyroxine to rats. Griesbach *et al.* (29) have found a ratio of 1.5:1 when *l*-thyroxine and *dl*-thyroxine were compared in their ability to prevent changes in the pituitary basophiles after methyl thiouracil. Moser (30) and Roche & Michel (31) have described color reactions for the microdetermination of thyroxine.

Harington (27), Rawson & McArthur (32) and Houssay (33) have reviewed various features of thyroid physiology and Reineke (34) has reviewed thyroactive iodinated proteins.

PARATHYROIDS

L'Heureux, Tepperman & Wilhelmi (35) have reported a new procedure for the preparation of parathyroid hormone which contains at least two components and no high molecular weight com-

pound. These authors (36) have also described a method for the estimation of parathyroid hormone activity by its effect on serum inorganic phosphorus in the rat. Tweedy, Chilcote & Patras (37), employing radiophosphorus, showed that the urinary and fecal excretion of injected phosphate decreased sharply in the first twenty-four hours following thyroparathyroidectomy. Within forty-eight hours the urinary and fecal excretion of administered radiophosphorus increased and fifteen to twenty-six days after the operation exceeded the control value. A reversal of the marked retention was obtained by administration of parathyroid extract. The prompt action of parathyroid extract is interpreted as evidence of direct effect upon the kidney. After bilateral nephrectomy, the administration of parathyroid extract had no effect upon the distribution, retention or excretion of radiophosphorus.

PANCREAS

Bouckaert & deDuve (38) have reviewed the evidence bearing upon the action of insulin and have concluded that primary importance must be assigned to the hepatic action of insulin. They have emphasized the importance of the blood sugar level in the interpretation of insulin effects and have suggested that many metabolic changes ascribed to insulin are produced solely by changes in concentration of glucose. Insulin by its action on those systems concerned with glucose phosphorylation permits the transformations of glucose to take place at lower blood sugar levels than would otherwise be possible.

Cori and co-workers have adduced evidence that a protein present in the anterior pituitary inhibits the hexokinase-catalyzed conversion of glucose to glucose-6-phosphate and that this inhibition can be overcome by insulin. Colowick, Cori & Slein (39) have shown that the hexokinase activity of muscle extracts from diabetic rats can be inhibited by the addition of adrenal cortex extract, whereas extracts from normal muscle and brain remain unaffected. The inhibition is dependent upon the presence of a very labile protein presumably of pituitary origin, since addition of suitable pituitary extract results in inhibition of phosphorylation either in the presence or absence of added adrenal cortex extract. Addition of insulin to either of these inhibited systems re-establishes normal phosphorylation; potassium hydroxide treatment of the insulin abolishes its effectiveness. The labile pituitary factor is not

identical with either purified adrenotropic, lactogenic or growth hormones. Krah1 & Cori (40) have extended these studies to intact muscle and have examined the uptake of glucose by the isolated diaphragm in normal, diabetic and adrenalectomized rats. Decreased utilization of glucose inversely proportional to the blood sugar rise was observed in severely diabetic animals; adrenalectomy restored the blood sugar to normal and raised glucose utilization in the diaphragm to the control rate. Addition of insulin to the isolated diaphragms of diabetic rats did not restore glucose utilization although the hexokinase content was found to be within the normal range. These results are complementary to those reported by Corkill & Nelson (41) who found that addition of insulin increased the glucose consumption and glycogen deposition in the isolated diaphragm from normal rats while treatment of the animals with anterior pituitary extract twenty-four hours prior to the experiment depressed the loss of glucose from the medium as well as glycogen deposition in the muscle; restoration of glucose consumption and glycogen deposition was not achieved by addition of insulin. In these experiments the prolonged action of the pituitary extract in the animal in contrast to its fleeting effect in the tissue extracts should be noted. Stadie & Zapp (42) have studied the factors which influence the use of the isolated rat diaphragm as a test object for the action of insulin. They have found increased glycogen synthesis roughly proportional to the amount of insulin added. The most striking feature with insulin present was the effect of potassium ion which in high concentration inhibited glycogen synthesis while the maximum glycogen deposition was found in the complete absence of potassium. The diaphragms from fed rats with a higher initial glycogen manifested an unimpaired ability to increase synthesis when insulin was added while the muscles from fasted rats ceased synthesis at a much lower glycogen level. Since there was no significant change in organic phosphorus during the period of glycogen synthesis, the authors believe that the extra ATP required for glycogen synthesis must be obtained from the reactions accelerated by insulin. These results were obtained without alteration of respiratory quotient or oxygen uptake, findings consistent with the observations of other investigators. Laszt & Vogel's claim (43) that the rate of glycogen phosphorolysis is increased in the muscles of alloxan-diabetic rats and was diminished by insulin was not confirmed by Staehelin & Voegtli (44) who

found that the reaction is depressed by insulin in normal rats and that there was no difference between the action of insulin on the tissues of normal or alloxan-diabetic animals.

Tiselius & Sanger (45, 46) found that insulin oxidized by performic acid was cleaved at the S-S linkages without effect on any of the amino acids except cystine. The presence of four components in the product was indicated by chromatography and electrophoresis. One of the fractions was shown to consist of a product containing essentially glycyl terminal residues, without phenylalanyl terminal residues, and lysine and arginine were not present. This product which appeared to be homogeneous by electrophoresis had a molecular weight of about 2500. Another fraction contained predominantly phenylalanyl terminal residues. The denaturation of insulin on standing was studied by Lens (47) and was found to be irreversible. It appeared possible that denaturation was followed by an oxidation. A neutral salt of the sulfuric acid ester of insulin in which nearly all of the aliphatic hydroxyl groups were involved was found to have a biological activity very similar to that of crystalline insulin by Glendening and co-workers (48).

The action of alloxan appears to be established as an irreparable destruction of the β -cells of the pancreas by a toxic agent which, while it affects other tissues, is rapidly destroyed and normal repair is possible. Goldner & Gomori (49) have confirmed their previous results that the secondary hypoglycemia following administration of the drug may be ascribed to the release of insulin from the degenerating β -cell and that if the pancreas is deprived of its blood supply for a short time during and after the injection of alloxan, there is neither subsequent hypoglycemia nor is diabetes established. Levey & Suter (50) have shown that administration of ascorbic acid immediately prior to alloxan increases the diabetogenic effect, and they believe the result is to be ascribed to the effect of ascorbic acid on the sulfhydryl groups of the animal thereby enabling more alloxan to reach the pancreas. Jiménez-Díaz *et al.* (51) reported that clamping the kidney pedicle prevented the diabetogenic action of alloxan, but this was denied by Gold (52) and Duff, Wilson & McMillan (53) who found that kidney circulation was not essential and ascribed the earlier result to reflex vasoconstriction in the pancreas. Bailey *et al.* (54) reported that dialuric acid was an effective diabetogenic agent in rabbits. Brückmann & Wertheimer (55) have investigated the structural specificity of

alloxan homologues in relation to diabetogenic activity, toxicity and amino acid and glutathione oxidation. They conclude that an intact pyrimidine nucleus is essential, substitution in one imino group diminishes activity and substitution elsewhere destroys the diabetogenic effect. Neither the deamination and decarboxylation of amino acids nor the oxidation of sulfhydryl groups can account for the action of alloxan. Burgen & Lorch (56) have attempted to determine whether alloxan interferes with cellular enzymes possibly involved in insulin synthesis. They found that alloxan was a reversible noncompetitive inhibitor of phosphatase and suggested that perhaps some of the effect may be the result of interference with zinc transfer in insulin synthesis. Houssay & Martinez (57) have investigated the effect of different diets on the sensitivity to alloxan and the development of pancreatic diabetes in the rat. The toxic and diabetogenic action increased with a low protein diet and even more so with a high fat diet. Thiamine or thiouracil added to the fat diets decreased the toxicity of alloxan and complete protection was observed with a diet high in coconut oil. A high lard diet resulted in greater diabetes following subtotal pancreatectomy and in these rats diabetes appeared first in the overfed groups, then in those which ate *ad libitum* and, finally, in those which were underfed.

Houssay, Foglia & Martinez (58) have summarized their studies of thyroid influence on diabetes in the rat. They have found that thyroidectomy or treatment with thiouracil greatly increased the dose of alloxan necessary to produce a diabetic state and simultaneous thyroidectomy and subtotal pancreatectomy or treatment of the partially depancreatized rats with thiouracil prevented the appearance of diabetes in a considerable percentage of animals. Thyroidectomy was without effect on incipient or manifest diabetes. Administration of thyroid for twenty days increased the sensitivity to alloxan, but after sixty days treatment the diabetogenic dose was slightly higher than for the control animals. In the partially pancreatectomized-thyroidectomized rat, correction of the thyroid deficiency brought an early appearance and rapid evolution of diabetes; when, however, the partially depancreatized animal was treated with larger doses of thyroid, the diabetes gradually disappeared. When incipient diabetes was treated with large doses of thyroid there was a temporary diminution but eventual reappearance of diabetes in one-third of the animals. The

authors have called attention to the different effect of thyroid in the dog where a diabetogenic effect is obtained.

Cantor *et al.* (59) found that in the permanent diabetes after alloxan, serum acid and alkaline phosphatase showed an initial and transitory fall. The acid phosphatase quickly returned to normal levels and remained unaltered subsequently. The alkaline phosphatase progressively increased over a period of two weeks to a value three times the normal level. Insulin returned the phosphatase level to normal and following cessation of this treatment there was a return to the diabetic value. Drabkin & Marsh (60) have reported increases in liver acid and alkaline phosphatase activity in diabetic rats while Foà *et al.* (61) have shown that insulin causes a rise in diphosphothiamine of dog blood either alone or when thiamine was injected.

Gaarenstroom (62) found a marked decrease in glucose excretion in alloxan diabetes following hypophysectomy but that administration of sugar raised blood glucose to a high level. The result was attributed to failure of appetite in the hypophysectomized rats, and in part, to a decreased rate of gluconeogenesis from protein. Ingle & Nezamis (63) showed that insulin did not delay the onset of glycosuria in rats force-fed a high carbohydrate diet but did permit the animals to tolerate larger amounts before they were killed by over-feeding. Winter (64) showed that prior fasting decreased the tolerance of rats to a force-fed high carbohydrate diet. Ingle *et al.* (65) attributed the decrease in glycosuria following diethylstilbestrol to a decrease in food intake since the compound caused exacerbation of diabetes in force-fed rats. Dohan & Lukens (66) have added evidence to support the view that sustained elevation of blood glucose leads to damage of islet tissue since hydropic degeneration of the islets and a permanent diabetes followed the prolonged administration of intraperitoneal glucose in normal and partially depancreatized cats. Greeley (67) from experiments on goats and rabbits concludes that the only apparent function of insulin in herbivora is that of mediating storage of carbohydrates.

Frame & Russell (68) and Ingle and co-workers (69) have studied the influence of glucose and insulin in the eviscerated rat. As in other species, there is a continuous rise of amino nitrogen independent of the blood sugar level. Insulin suppresses this rise and the effect is quantitatively proportional to the dose and not to the

glucose level. The former authors found that the administration of insulin together with anterior pituitary extract, which itself has no effect in the eviscerated rat, gave a greater diminution than insulin alone. Friedberg and Greenberg (70) in an investigation of the endocrines in the regulation of amino acid level in blood and tissues showed that insulin administration decreased the blood amino acid concentration in normal rats.

Anderson *et al.* (71) have described a micromethod for the detection of insulin in blood using an adrenal demedullated diabetic hypophysectomized rat. The test was sufficiently sensitive to establish that when an isolated pancreas is perfused with blood of low glucose content, insulin is not secreted in detectable amounts, whereas a high glucose level in the perfusate stimulates insulin secretion (72). Young & Lewis (73) have described an improved method for conducting the mouse assay of insulin, and Lacey (74) has published a critical analysis of the rabbit assay of insulin.

GONADS

Methods for separation of steroid metabolites from tissues have been described by Samuels (75) and the enzymatic destruction of testosterone by several tissues from different species has been investigated by his group (76). The α - β -unsaturated ketonic linkage was eliminated without evidence of the formation of 17-ketosteroids or other carbonyl containing compounds. Clark & Kochakian (77) have shown that rabbit liver slices converted testosterone to Δ^4 -androstene-dione-3,17 and *cis*-testosterone; other partially identified products including one which contained three oxygen atoms were present. These results suggest the following equilibrium existed:

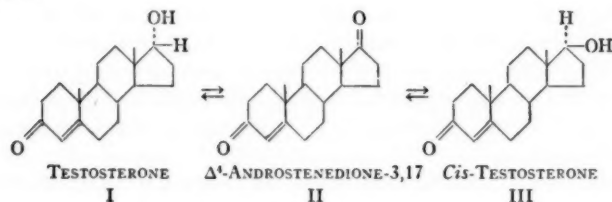


FIG. 1

No evidence was obtained for the presence of known urinary androgens or estrogens but the authors point out that not only may

other tissues be involved but that the metabolism may be different in the rabbit than in other species. Lehninger & Scott (78) could not find any net increase or decrease of estrogenic activity when estrone or α -estradiol was added to human prostate slices nor did these substances affect the respiration of the slice. The authors called attention to adsorption of the steroid on the tissue, a non-metabolic process since heat-inactivated slices showed identical behavior. After administration of dehydroisoandrosterone acetate to human subjects with Addison's disease, Mason & Kepler (79) isolated androsterone, etiocholanolone, Δ^6 -androstene-3, (β) 16, 17 triol and a nonketonic $C_{19}H_{22}O_2$ compound from the urine and drew the conclusion that dehydroisoandrosterone is not necessarily an end product of steroid metabolism. These authors offer an explanation for the greatly increased β -hydroxy steroid excretion with adrenal cortical tumors in contrast to the relatively low production of these substances in adrenal hyperplasia. In both instances overproduction of dehydroisoandrosterone is assumed; with hyperplasia the capacity of the body to destroy or convert the intermediate is not exceeded, whereas so great is the excess from tumors that the substance is excreted in large amounts. Fishman (80) has made the interesting suggestion that β -glucuronidase is involved in the metabolism of estrogenic hormones. The *in vitro* conjugation of estrogens with glycuronic acid by liver slices has been studied by Crepy (81).

While the capacity to inactivate estrone *in vitro* is lost in the livers of rats with Vitamin B deficiency, Zondek & Finkelstein (82) have shown that this ability still remains at a high level in the intact rat. Hooker *et al.* (83) have shown that monkey liver is relatively inefficient in the inactivation of estrogen *in vivo*. Klatskin *et al.* (84) have studied the gynecomastia observed in American prisoners of war held at Japanese prison camps for long periods. It was suggested that malnutrition either impairs the ability of the liver to inactivate estrogen or depresses testicular function with decrease in androgen, leading to gynecomastia. Salter *et al.* (85) studied the output of urinary 17-ketosteroids, cortin, estrogen and follicle-stimulating hormone (FSH) in these subjects. As a group the gynecomastics showed a significantly lower excretion of 17-ketosteroids than did comparable control subjects while the estrogens were in the low, normal range. Cortin and FSH were essentially normal. These results appear to favor the

inhibition of testicular function rather than an impaired ability of the liver to detoxicate or to inactivate estrogens. Roberts & Szego (86) believe that accelerated estrogen activation is responsible for the enhanced effect of α -estradiol on the uterus observed with liver regeneration after partial hepatectomy in rats. These authors (87) had previously found that estrone was ineffective in the eviscerated rat. The numerous other functions of the liver must be considered in the interpretation of these experiments. Segaloff (88) concluded that progesterone reduced the amount of α -estradiol inactivated by the liver *in vivo* and simultaneously decreased the peripheral effectiveness of the estrogen. Davis & Fugo (89) have shown that diethyl stilbestrol and testosterone did not alter pregnanediol excretion in pregnant humans while 30 to 35 per cent of administered progesterone was recovered as pregnanediol.

Gordan *et al.* (90) have studied the effect of testosterone propionate on nitrogen retention and body weight in normal and hypophysectomized rats. Since both groups exhibited nitrogen retention and only the intact rats made appreciable weight gains, it was concluded that the utilization of the retained nitrogen differed in these two groups of animals. However, since the operated animals also gained weight, it is apparent that testosterone does not act solely through the pituitary. Davies & Mann (91) have shown that fructose which is the normal nutrient of spermatozoa (92) accumulates in the accessory sex glands before the appearance of spermatogenesis, and Mann & Parsons (93) have shown that the fructose content of the accessory sex organs is under the influence of testosterone. This "fructose test" is a sensitive reaction which can be conveniently applied for the detection of testicular hormones since the post-castration fall and the increase with testosterone occur rapidly. Atkinson & Elftman (94) have observed a marked increase in uterine alkaline phosphatase following estradiol administration in the mouse and suggest that the mobilization of alkaline phosphatase is a link in the mechanism by which estrogen affects lipid and carbohydrate metabolism in the uterus. Similar results were reported for monkeys and humans (95). The level of acid phosphatase in the serum was correlated with androgen excretion in the urine by Engberg *et al.* (96), but androgen or estrogen administration produced erratic responses. Lundquist (97) found that prostatic phosphatase readily splits phosphoryl choline, the presence of which was shown in semen. McShan *et al.* (98) have reported that the phenolic group of various estrogens inhibited the

succinoxidase system of rat tissues without destruction of the biological activity of the hormones whereas no inhibition was observed with the sulfuric acid esters of androgens. Buchwald & Hudson (99) have found that diethylstilbestrol and testosterone propionate administered to hypophysectomized rats were without effect on the serum calcium, phosphorus or alkaline phosphatase activity. Diethylstilbestrol in males decreased the acid phosphatase of the blood serum while testosterone propionate in females had no effect. Estrone has been shown by György, Rose & Shipley (100) to exert lipotropic action and to augment that of methionine.

Meyer *et al.* (101) have measured a progressive increase in succinic dehydrogenase activity of the corpus luteum of pregnancy in rats to the eleventh day of pregnancy after which the value remains essentially constant but decreases rapidly at parturition. The authors believe that the increase is associated with increasing production of progesterone and possibly other ketosteroids. The succinic dehydrogenase of the corpus luteum of the postpartum ovulation increased in concentration to a maximum at the twentieth day of lactation and was then greater than that in the corpus luteum of pregnancy. The residual ovarian tissue varied little during pregnancy or lactation. Stafford *et al.* (102) have made similar studies of the phosphatase activity of the corpus luteum. The evaluation of these changes with our present meager knowledge of the biochemical function of the corpus luteum is extremely difficult. Everett & Sawyer (103, 104) have studied the serum cholinesterase in rats and have found that it appears to parallel the estrogen level. Castration in the female resulted in a decline while in the male a gradual rise ensued; estradiol produced an elevation while testosterone caused a decrease in activity. Progesterone was without effect.

Prelog *et al.* (105) have isolated 3(α)- and 3(β)-allopregnanolone-20 together with Δ^5 -pregnenol-3(β)-one-20 from pig testes and were able to demonstrate the presence of testosterone in the extract by infrared spectroscopy. In addition to the C_{21} steroids three C_{27} compounds, namely $\Delta^{3,5}$ -cholestadienone-7, Δ^5 -cholestenol-3(β)-one-7 and 3,6 cholestanedione have been isolated from extracts of this gland. While the two unsaturated sterols are probably artefacts formed during isolation, the cholestanedione which was present in higher concentration is considered to be a normal constituent by the authors.

It is interesting to note that the saturated steroids isolated

from grandular sources thus far have been exclusively derivatives of the allocholane series (106). This would indicate that such derivatives are intermediates in steroid hormone synthesis or exercise some hormonal action as yet not understood. As a suggestion, it has been shown by Nelson & Merckel (107) that androstanedione has a more efficient action in the prevention of testicular tubular degeneration following hypophysectomy than does testosterone. In view of the probability (*vide infra*) that this localized action is a true hormonal mechanism the possibility certainly exists that it is mediated by one of these allocholane derivatives. Accordingly some biochemical role for the allocholane derivatives of the adrenal should be sought and the possibility that allocholane derivatives in general participate in metabolic processes should not be disregarded.

Allopregnanediol-3(β), 20(β) was isolated from ox bile by Pearlman (108) and attention was drawn to the route of excretion of a C_{21} steroid and to the configuration at C_{20} since the pregnanediol isolated from urinary sources possesses a C_{20} (α) hydroxyl group. Estrone appears to be the principal estrogen in the bile of pregnant cows from the studies of Pearlman *et al.* (109) and is present in amounts of 0.6 mg. per liter most of which appears in the free form. Etiocholanol-3(α)-dione-11,17 and Δ^9 -etiocholenol-3(α)-one-17 were isolated from human urine by Lieberman, Dobriner and their associates (110, 111). The unsaturated compound, probably a dehydration product of the corresponding 11(β) dihydroxy compound, appears to be associated with neoplastic disease and is found very rarely in the urine of presumably normal individuals. The adrenal origin of these products is almost certain from the presence of the C_{11} oxygen function and the metabolic transformation of the characteristic C_{21} adrenal steroids to a C_{19} excretion product is strongly suggested from these results. These compounds might, nevertheless, be derived from adrenosterone. The problem then of the conversion of a C_{21} steroid to a C_{19} compound, which has been repeatedly postulated, should be critically investigated especially since Hirschmann & Hirschmann (112) have recently suggested² that Δ^5 pregnenediol-3(β), 17(α)-one-20 and dehydroisoandrosterone are mutually interconvertible in the body. These workers isolated the two aforementioned substances together with³ 3(β)-chloro- Δ^5 -androstenone-17 from the urine of a boy with ad-

² Configuration altered from original to conform with (176).

³ Configuration altered from original to conform with (201).

renocortical carcinoma. In addition they isolated and identified 17 α -methyl- Δ^5 -D homoandrostenediol-3(β), 17 α (α)-one-17, an artefact formed from pregnenediolone in the course of isolation. Sutherland & Marrian (113) have succeeded in preparing pure sodium pregnenediol-3(α)-20 (α)-glucuronide from the impure substance isolated from urine and generally designated by that term. The substance usually isolated was shown to contain about 20 per cent contaminant since from the ketonic fraction, prepared under special conditions from the impure product, they obtained the hitherto undescribed sodium pregnanol-3(α)-one-20 glucuronide.

Shoppee (114) has called attention to the high degree of specificity exhibited in the biological action of diastereoisomers at C₁₇ and has indicated that the mode of union of the C and D rings in estrone and equilenin is not beyond doubt although the configuration of the other steroid hormones appears to be established. Furchgott, Rosenkrantz & Shorr (115) have continued their studies of the infrared absorption spectra of steroids and have discussed the relationship of certain bands to the chemical structure of progesterone and various derivatives of pregnane. Huffman (116) has achieved the partial synthesis of estriol from estrone in 22 to 26 per cent over-all yield and has described (117) the preparation of 16-ketoestrone methyl ether and 16-ketoestradiol methyl ether. The bioassay of three diastereoisomers of estriol in both immature and castrated adult rats is reported by Huffman & Grollman (118) and estriol was found to be the most active. 16-Ketoestrone and 16-keto- α -estradiol were inferior to estriol; estradiol was more active in both tests, while estrone was slightly more active in the adult castrate but less potent in the immature rat. The authors have discussed the formation of estriol in the body and favor the view that estrone is oxidized to 16-ketoestrone which is converted to estriol with intermediate formation of 16-keto- α -estradiol according to the following partial formulation. Wilds & Djerassi (119) have confirmed the partial synthesis of α -estradiol from cholesterol earlier reported by Inhoffen & Zuhlsdorff (120). Turner (121) has described the preparation of C¹⁴ 3- radiotestosterone.

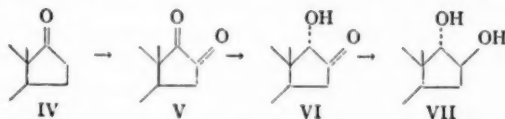


FIG. 2

A fluorometric method for the determination of estrogen for which a high degree of sensitivity is claimed is described by Jailer (122) and by Finkelstein *et al.* (123). On the other hand, Bender & Wilson (124), who investigated and rejected the method of Talbot *et al.* (125), believe that no satisfactory chemical method is available for urinary estrogens, especially when small amounts are present; they suggest that bioassay is the only reliable method. The polarographic determination of several steroids has been studied by Sartori & Bianchi (126), Björnson *et al.* (127) and by Barnett and co-workers (128, 129, 130). Johnston (131) has tested the chromatographic separation of steroid dinitrophenylhydrazones. The bioassay of estrogens by intravaginal implantation of pellets has been studied and criticized by several investigators (132, 133, 134). Its application without careful control would seem of dubious significance. A sensitive bioassay for progesterone by direct application in the mouse uterus has been reported by Hooker & Forbes (135). Curtis *et al.* (136) have prepared a graph and nomograph for the statistical evaluation of estrogenic assays. Methods for the preparation of relaxin from sow corpora lutea residue and from whole ovaries have been described by Albert and co-workers (137). Hall & Newton (138) have described the synergistic action of estrone and crude relaxin on the symphysis pubis of spayed mice and have suggested that the placenta stimulates relaxin secretion by the ovary.

The spermatogenic action of testosterone in hypophysectomized rats has been investigated by Simpson & Evans (139), and it was found that a stimulation of spermatogenesis was obtained only at dosage levels which were effective in stimulating the male accessories. In contrast, ICSH (interstitial cell stimulating hormone) from the pituitary was effective in dosages far below those which produced changes in the accessory organs. It appeared that the local concentration of the hormone in the gonad might account for the discrepancy and the authors accept, although with reservation, the view that ICSH is a complete gonadotropic hormone in the male. Chu & You (140) have extended these studies to the hypophysectomized pigeon and find that testosterone is capable of maintaining full spermatogenesis in the atrophic testes, a reaction which does not take place in the presence of pituitary tissue. In female birds testosterone permitted follicular growth while estrogen was without effect. This latter result is in conflict with the work of Williams (141) in the hypophysectomized rat where follic-

ular degeneration was prevented by estrone and stilbestrol implants. Masson (142, 143) has studied several steroids for spermatogenic action in testes of hypophysectomized and estradiol treated animals. Especially noteworthy is the effect of pregnenol 3(β)-one-20 since this substance has been shown to be a testicular constituent. Masson states that the compound exerts a "passive" effect in that it can protect the germinal epithelium and maintain it at the stage at which hypophysectomy was performed in contrast to a steroid like testosterone or the compound⁴ 17-methyl-androstenediol-3(β), 17(β) which are "active" spermatogenic agents in that they can effect repair. These various results would appear to leave little doubt that the gonad hormones play a significant part in the economy of the organ which produces them.

Hisaw (144) has reviewed the development of the Graafian follicle and ovulation, Kochakian (145) has reviewed the protein anabolic effects and (146) the role of enzymes in metabolic activities of steroid hormones.

ADRENAL

The hormones of the adrenal cortex regulate the distribution of electrolytes between tissues and fluids and influence the rate of gluconeogenesis from protein. They are intimately involved in the reactions by which the body maintains itself when subjected to stress. Long (147) has suggested that the hormone is synthesized from cholesterol stored in the gland and that in these processes ascorbic acid disappears. Despite the wealth of information about the steroid constituents of the gland it is unknown whether one or more substances are secreted into the blood stream and there is considerable evidence that some constituent of the "amorphous fraction" may have physiological importance other than those ascribed to the known steroid constituents. Many of the adrenocortical steroids have been prepared by partial synthesis.

White & Dougherty (148, 149) have drawn attention to the lymphocyte as a cell specifically influenced by the adrenocortical steroids. Dissolution or involution of lymphoid organs is brought about by cortical steroids and the protein from these structures is available for other purposes. One of these proteins is identical with the normal serum gamma globulin and the effect of the cortical steroids is stated thus indirectly to increase serum globulin. Al-

⁴ Configuration altered from original to conform with (176).

though it was reported (150) that an increased antibody production resulted and that simultaneous administration of antigen and adrenocortical extract produced higher antibody titers, the effect has been denied by Eisen *et al.* (151). These authors concluded that adrenal cortical activity was not essential for the fabrication or release of antibodies and gamma globulin since adrenalectomized rats maintained with salt and desoxycorticosterone exhibited antibody titer and gamma globulin levels identical with similar rats receiving adrenal cortical extract during immunization. The result could not be ascribed to increased turnover of serum protein since after feeding glycine labeled with N^{15} replacement of the isotope in the proteins occurred at the same rate in both series of animals.

White & Dougherty (152) have studied the role of the adrenal cortex and the thyroid in the mobilization of nitrogen from tissues in the fasting mouse. The fasting adrenalectomized mouse drew significantly on the carcass and liver for its nitrogen demands while the lymphoid tissue remained relatively unaffected, whereas normal mice, subjected to the same fast, lost nitrogen from lymphoid tissue as well. From a relative standpoint the contribution of the lymphoid tissue was greatest, but the greatest absolute loss was from the carcass. The fasted thyroidectomized mouse obtained significant quantities of nitrogen only from liver and lymphoid tissue while the adrenalectomized-thyroidectomized mouse was able to obtain nitrogen from liver only. The thyroid therefore controlled the rate of catabolism in muscle tissues, and the adrenal cortex controlled the breakdown of lymphoid tissue. This conclusion was affirmed by administration of adrenal cortical extract to the fasted mice which resulted in greater loss from the lymphocytes without any additional loss from other tissues. The authors suggest that adrenal cortical secretion is one of the factors regulating thyroid activity inasmuch as the loss of carcass nitrogen was greater in fasted adrenalectomized mice treated with adrenocortical extract. The reverse relationship, i.e., augmentation of adrenal activity by thyroid secretion, does not obtain since maximal involution of lymphoid tissue can be found in the absence of the thyroid.

The role of the adrenal in the mobilization of protein has been studied by Berman *et al.* (153) who have reported that adrenalectomy inhibits the deposition of protein in the remnant and prevents the increase in fat and water content of the regenerating liver fragment after partial hepatectomy. Adrenal cortical extract substitutes for this function of the adrenal whereas desoxycorticosterone

was ineffective. To provide an index of the time required for hormonal action Ingle *et al.* (154) have examined the effect of adrenal removal on the survival time of eviscerated rats and found that survival is decreased. The effect is manifest within two hours but whether cortex or medulla was responsible was not determined. Ingle *et al.* (155) found that adrenalectomy was without effect on nitrogen excretion in either the force-fed or the fasted rat and that therefore the adrenal was not essential for the adjustment of nitrogen balance. Cowie & Folley (156, 157) found that adrenalectomy in rats on the fourth day of lactation caused a marked inhibition of milk production and that lactation could be partially maintained with desoxycorticosterone acetate. 11-Dehydrocorticosterone was less effective than desoxycorticosterone in maintenance of lactation in adrenalectomized rats on a high protein diet.

Venning (158) has followed the urinary excretion of cortical hormone metabolites during human pregnancy using the deposition of glycogen in the liver of adrenalectomized mice as the assay procedure (159). An initial rise during the first third of pregnancy was followed by a fall and then a marked secondary rise. During the last month the excretion again diminished and low values were found after parturition. Ludewig & Chanutin (160) have studied adrenal cholesterol and ascorbic acid after injury. Thermal burns produced a marked fall in cholesterol esters during the first twenty-four hours and a persistent and marked increase subsequently. Ascorbic acid followed a similar pattern. Administration of tris-(β -chloroethyl) amine and di-(β -chloroethyl) sulfide resulted in increases of cholesterol and ascorbic acid after twenty-four hours, results attributed to adaptation syndrome after injury.

Folley & Greenbaum (161) have found that the decrease in liver and mammary gland arginase which follows adrenalectomy is not the result of anorexia since operated animals exhibited a much greater fall in arginase activity than pair-fed controls. Kochakian & Vail (162) found that administration of adrenal cortical extract did not elevate the liver arginase activity in adrenalectomized rats but did partially restore kidney arginase, although there was a marked increase in urinary nitrogen and liver glycogen with the extract. Previous treatment of the adrenalectomized rats with testosterone propionate did not alter the effects of adrenal cortical extract but greatly increased kidney arginase. The same workers (163) reported that liver alkaline phosphatase activity is slightly increased in the rat following adrenalectomy but is greatly

augmented following hourly injections of adrenal cortical extract. The enzyme activity responded faster than the deposition of liver glycogen. Desoxycorticosterone was ineffective as was also prior treatment with testosterone propionate. The alkaline phosphatase of kidney is decreased slightly following adrenalectomy but this can be prevented by administration of sodium chloride or desoxycorticosterone acetate. Adrenal cortical extract was without effect on this enzyme. None of the foregoing experimental procedures affected the acid phosphatase.

Pabst *et al.* (164) have compared four pure compounds and two extracts, measuring liver-glycogen deposition in adrenalectomized rats and muscle work performance. The order of activity was the same by either method. 17-Hydroxycorticosterone was more active than the 11-dehydro derivative while corticosterone and 11-dehydrocorticosterone were much less active. Any one of these substances can serve as a reference standard. Hog adrenal extract was twice as potent per unit weight of gland as a corresponding beef adrenal extract.

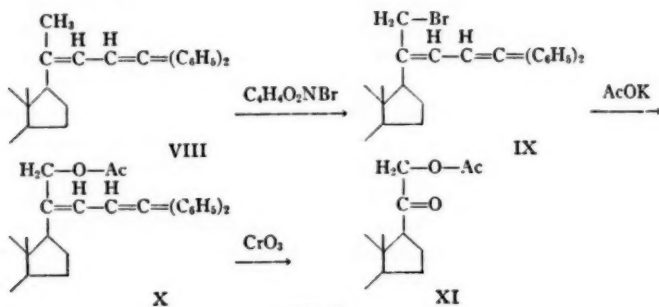


FIG. 3

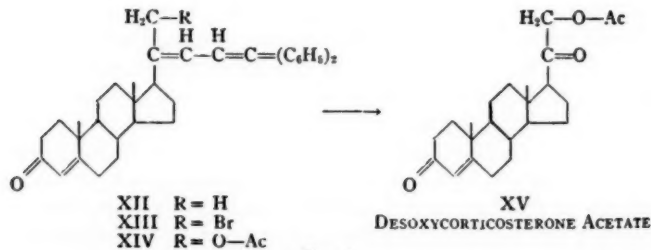


FIG. 4

Perhaps the most important recent advance in the partial synthesis of adrenal steroids is the contribution of Meystre & Wettstein (165) who have devised a greatly simplified procedure for the preparation of the ketol side chain of the cortical steroids from the bile acids. The reactions involved are summarized in Fig. 3. The bromination of a $\Delta^{20,23}$ -24,24-diphenylcholadiene (VIII) (166) with N-bromosuccinimide proceeds readily and the product is relatively stable. The halogen can be replaced with hydroxy, acyloxy (IX) or alkoxy derivatives and these products oxidized with chromium trioxide to the corresponding 20-ketopregnanes (XI). Similar reactions can be conducted when nuclear ketonic groups are present or in the presence of nuclear unsaturation (167). Especially interesting is the direct conversion of XII through XIV to desoxycorticosterone acetate (XV). The same authors (168) have extended these reactions to 11-keto bile acids and have in this way prepared 11-dehydrocorticosterone acetate.

Turner *et al.* (169) have described the introduction of an oxygen function at C₁₁ of the steroid nucleus over the unsaturated 3,9-epoxide (170). Silver oxide converts one of the diastereoisomeric

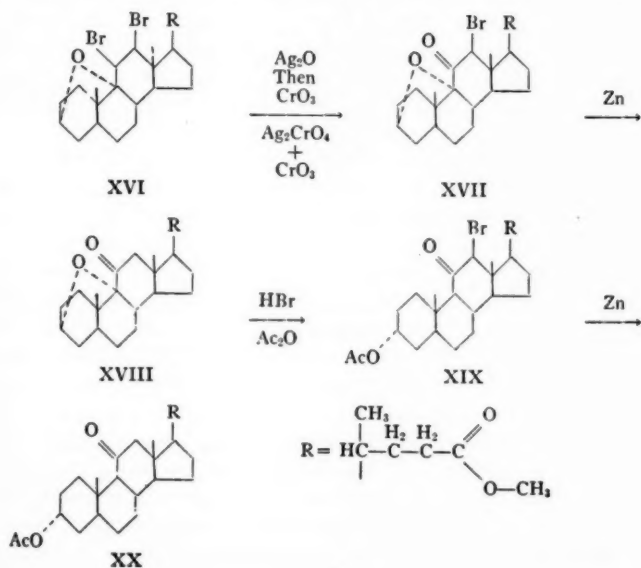


FIG. 5

dibromides XVI of this structure to an 11-hydroxy-12-bromo derivative which, following oxidation, yields the 11-keto-epoxide (XVII) after treatment with zinc. Cleavage of the oxide ring was accomplished with HBr in acetic anhydride to yield a 3(α)-acetoxy-11-keto-12-bromo derivative (XIX), a rearrangement product of the anticipated 9-bromo compound. Reduction with zinc completed the preparation of the 11-ketone (XX). Sarett (171) has described the partial synthesis of dehydrocorticosterone acetate from a mixture of Δ^{17} - and Δ^{20} -pregnenedione-3,11.

Velluz *et al.* (172) have studied the saponification of ketol acetates and have found that approximately correct values are obtained in the absence of oxygen. Reich & Lardon (173) and Shoppee (174) have prepared Δ^9 -androstenol-3(β)-one-17, and Lardon & Lieberman (175) have made Δ^{11} -etiocholenol-3(α)-one-17 for comparison with products isolated from urine. Von Euw & Reichstein (176) have reviewed the evidence bearing upon the configuration at 3,11 and 17 positions in the naturally occurring cortical steroids. Their conclusions may be summarized: (a) that with a single exception, where a 3 hydroxyl group, or (b) an 11 hydroxyl, or (c) a sidechain is present, it is in β configuration; and (d) that when both a sidechain and hydroxyl group are situated at 17 the hydroxyl group very probably is in α configuration. Thus, for example, the structure of corticosterone and 17-hydroxycorticosterone would be represented as follows:

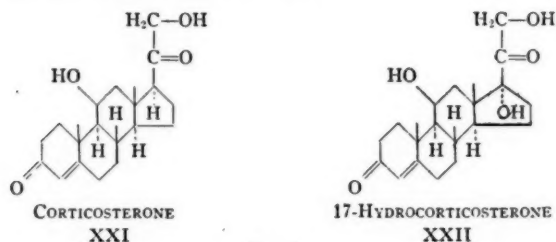


FIG. 6

Gurin & Delluva (177) have found that phenylalanine is a precursor of epinephrine in the rat since the amino acid labeled with C^{14} in the carboxyl group and α carbon was converted to epinephrine in which the C^{14} was localized in the terminal carbon of the sidechain. Similar results were obtained with tritium-labeled phenylalanine.

Long (147) has reviewed the relation of cholesterol and ascorbic acid to adrenal secretion, and Pincus (178) has discussed the role of the adrenal in human stress. Kendall (170) and Gallagher (179) have reviewed certain aspects of the partial synthesis of cortical steroids.

PITUITARY

Fishman (180) has described a simplified method for the preparation from hog pituitaries of adrenocorticotropic (ACTH) hormone which behaved as a single component electrophoretically and appeared to be pure from solubility studies. Two interesting reports of the administration of ACTH in man have appeared. Mason *et al.* (181) found that when 25 or 50 mg. per day were given to a normal young woman on a constant diet no significant change occurred save an increase of cortin-like substance in the urine. When the dose was increased to 100 mg. per day there was an increased excretion of both cortin-like material and 17-ketosteroids. This was considered definite evidence for stimulation of the cortex since the values quickly reverted to the control following cessation of treatment. A slight increase in urinary nitrogen, a decreased excretion of creatine and a pronounced decrease in hemoglobin were observed. No significant alteration of other blood and urinary constituents was found. Thorn *et al.* (182) who administered a less highly purified preparation of the hormone to a man with secondary adrenal cortical insufficiency noted a similar pronounced increase in "11-hydroxy steroids" and 17-ketosteroids. In addition there was a marked increase in urinary nitrogen with a concurrent rise in uric acid excretion. Creatinine remained constant through the experimental period while sodium excretion was markedly decreased. In view of the nitrogen excretion together with sodium retention the authors suggested that 11-oxygenated steroids, known to promote catabolism of protein, were secreted together with substances of the desoxycorticosterone type, which cause electrolyte retention. These authors have pointed out that the increased 17-ketosteroid excretion may be the result of higher output of C_{21} steroids by the adrenal followed by oxidative removal of the side chain or stimulation of the gland to elaborate C_{19} ketosteroids and related compounds.

Bennett *et al.* (183) have administered ACTH to rats made diabetic with alloxan and maintained on a carbohydrate-free diet. The glycosuria and nitrogen excretion were enhanced by the hor-

mone and the extra nitrogen was not sufficient to account for the increased glucose excretion. The authors conclude that ACTH, presumably through the adrenal hormone, not only increases gluconeogenesis from protein but likewise depresses carbohydrate utilization. Sayers & Sayers (184) have measured the changes in adrenal ascorbic acid as a means of studying ACTH secretion in rats. Exposure to stress of various types was followed by diminution of ascorbic acid and this could be prevented by administration of biologically active cortical steroids. By determining the amount of ACTH required to produce a fall in adrenal ascorbic acid comparable to that produced by a given stress, the authors obtained a measure of the ACTH secretion of the animal's own gland. Since pretreatment with cortical steroids did not affect the response to injected ACTH the conclusion was drawn that the steroids were the effective agents in the suppression of pituitary secretion and that these steroids did not suppress the adrenal gland directly. They determined that the degree of inhibition was proportional to the amount of steroid administered and that with greater stress more cortical hormone was required for suppression of pituitary secretion. Their results were interpreted to indicate that ACTH is elaborated at a rate inversely proportional to the concentration of cortical hormone in the body fluids in accordance with the steroid requirements of the peripheral tissues. They suggested that the steroids act directly on the pituitary rather than through products of their metabolic activity.

Jungck *et al.* (185) have examined the problem of regulation of pituitary secretion by the other endocrine organs with somewhat different methods and have reached an opposite conclusion. Auto-transplants of both ovaries to the spleen were made in rats so that the estrogens were secreted into the portal circulation and were inactivated in the liver as evidenced by atrophic uteri and vaginae and hypertrophic thymus glands. The pituitaries of these rats were then assayed for their gonadotropic content by maceration and transplantation to immature female rats; comparison was made with the pituitary gonadotropic content of normal and castrate rats. The pituitaries from the rats with splenic ovarian transplants showed a gonadotropin content somewhat elevated from the normal gland but not comparable to the greatly elevated gonadotropin of the castrates. It was therefore concluded that the ovaries normally inactivate gonadotropin and the rise following castration is the result of failure of this inactivation. Inhibition by estrogen

has a minor role in pituitary control except when large unphysiological doses are administered. Heller & Jungck (186) have examined the ovarian weight after intrasplenic transplantation and found a threefold increase after thirty to fifty-seven days over that at transplantation. Since the gonadotropin content of the pituitary in these rats was only slightly increased, the hypertrophy of the ovaries was ascribed to the low content of estrogen in the circulating blood. When estradiol benzoate was given to the experimental rats in doses of 0.5 to 5.0 $\mu\text{g.}$ per day the weight of the ovary was suppressed to a lower value than prior to transplantation. The conclusion was therefore drawn that ovarian growth is inhibited by circulating estrogens.

It is to be noted that Jungck *et al.* have measured not the secretion of the pituitary but the hormone stored in the gland. While this may be an indirect measure of the level of the circulating hormone it is obviously necessary to have a more precise criterion of the pituitary secretion reaching the stimulated gland. Further, since it is unquestioned that estrogen can inhibit the pituitary, it is pertinent to explore what can be considered a large and unphysiological dose since there are different threshold levels for the several effects of the hormones. In the virtual absence of information about the secretory rate of any gland except the thyroid, a precise evaluation of the factors involved in hormonal interrelation must remain largely speculative.

Hertz & Meyer (187) have attempted to determine the time relationship between pituitary secretion and ovulation in the rat. When hypophysectomy was performed in early proestrus no ovulation ensued and it was concluded that there was not sufficient circulating hormone to produce the reaction. The quantity of gonadotrophin therefore necessary to produce ovulation and luteinization must be secreted subsequent to this phase of the cycle. Tepperman & Tepperman (188) have studied the action of gonadotropic hormones on the free and esterified cholesterol of the testes in order to determine whether these substances participate in metabolism in a manner analogous to that ascribed to the adrenal. It was concluded that the fluctuations in esterified cholesterol were affected by the gonadotropins. "FSH" produced an increase in cholesterol ester and comparatively small changes in testis weight; "ICSH" did not alter the cholesterol ester but produced marked seminal vesicle increase; treatment with "FSH" followed by "ICSH" produced a striking fall in cholesterol ester associated

with increased seminal vesicle weight. It was suggested that ICSH may function in the conversion of cholesterol ester to androgen.

Maddock & Heller (189) have found that rat and sheep gonadotropin were inactivated by shaking for two and one-half hours at 37° while only slight inactivation could be detected at 3° or after incubation of rat gonadotropin for two and one-half hours at 37°. The inactivation could be prevented by addition of whole blood, egg white, etc. Franklin *et al.* (190) have reported the amino acid composition of an electrophoretically pure growth hormone preparation from beef glands by microbiological procedures. The molecular weight was estimated to be 46,800, based on the approximate molecular weight from osmotic pressure determination and from the histidine content (2.65 per cent) assuming eight histidine molecules per mole. Li & Fraenkel-Conrat (191) have studied the esterification with methanol of the carboxyl groups of lactogenic hormone. The protein became more basic through esterification and the homogeneity of the product was comparable to the initial preparation. Assayed in the squab, the biological activity diminished progressively with the introduction of methoxyl groups into the molecule and it was concluded that the carboxyl groups are essential for lactogenic activity. Meites & Turner (192) have found that the secretion of lactogenic hormone in the pigeon, unlike that of the mammal, is not stimulated by the administration of estrone, progesterone or testosterone.

Li & Reinhardt (193) have found that administration of ACTH or growth hormone increased the albumin-globulin ratio in the plasma of hypophysectomized rats, an increase dependent on a rise in plasma albumin. There was no increase in the globulin fraction following treatment with ACTH in either normal or hypophysectomized rats. The cervical and thoracic duct lymph contained four components with mobilities similar to those found in plasma. The protein pattern in the cervical duct lymph was unaltered by treatment of the animals with ACTH. Ingle *et al.* (194, 195) have administered ACTH to male rats force-fed high carbohydrate, high fat and high protein diets in isocaloric amounts. Some of the rats on the high carbohydrate diet developed glycosuria during treatment; all excreted more nitrogen, most marked with the high fat diet and least with the high protein. All animals excreted more potassium; changes in sodium chloride excretion were irregular but there was no evidence of sodium retention. There was striking hypertrophy of the adrenal and atrophy of the thymus.

Gordan *et al.* (196) found that administration of growth hormone to rats fed a six per cent casein diet resulted in nitrogen retention without weight gain. Supplementation with methionine permitted the diet to support the rapid growth induced by the hormone. Li *et al.* (197) have shown that growth hormone produced a marked increase in plasma alkaline phosphatase of normal and hypophysectomized rats which could be counteracted by ACTH. Anderson & Long (198), from experiments with the isolated pancreas, have concluded that growth hormone inhibits insulin secretion by direct action on the islet cells. Neither adrenal cortical hormones nor thyroxine inhibit insulin secretion. These results do not support the concept of a pituitary pancreatropic hormone but do not exclude a diabetogenic action of other pituitary factors on extra pancreatic tissues.

Samuels (199) has reviewed the relation of anterior pituitary hormones to nutrition and Thayer (200) has reviewed the bioassay of pituitary and adrenal hormones.

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THE VITAMINS¹

BY BERNARD L. OSER

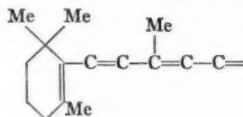
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As the years go by and proof of the existence of new vitamins becomes more difficult and hence less frequent, one might expect a decline in the number of reports of research in this field. Such, however, is not the case. Indeed the plight of the reviewer in selecting a sufficiently small number of published papers to cover adequately the advances of the past year within limited space is not an enviable one. Of necessity, many worthy researches must here remain unhonored and unsung, including scores of papers that have appeared in 1947 on the occurrence and distribution of vitamins in foods, the effects of cooking, preservation, and storage conditions on vitamin retention, the advances in vitamin fortification, etc.

Though dietary considerations justify continuation of the practice of grouping the vitamins together as a special class of nutrients, from the standpoint of either organic chemistry or physiology the substances bear little structural or functional resemblance to each other. Nevertheless, for the sake of convenience, the precedent of discussing the vitamins as a group will be continued.

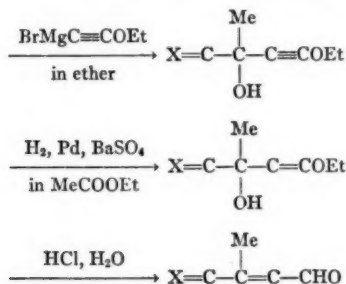
VITAMIN A

Synthesis.—In the *Annual Review of Biochemistry* for 1947, Embree (1) referred in detail to the synthesis of various vitamin A ethers and esters by Milas, Oroshnik, Isler *et al.*, Kanner *et al.*, Cymerman *et al.*, van Dorp & Arens, and Karrer. Van Dorp & Arens (2) have now reported a synthesis of vitamin A aldehyde and also of vitamin A. These workers have acknowledged the aid of Dr. H. H. Inhoffen, who directed their earlier work in the synthesis of vitamin A acid, and have now independently modified the original synthesis to obtain the aldehyde and alcohol, starting with C₁₈ ketone [compound XVII in Embree's review (1)]. Writing X for



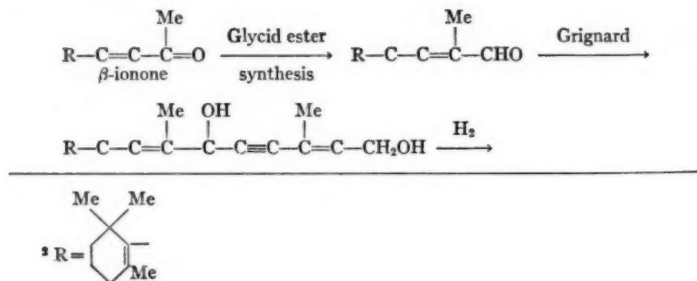
¹ This review covers the period from November, 1946 to December, 1947.

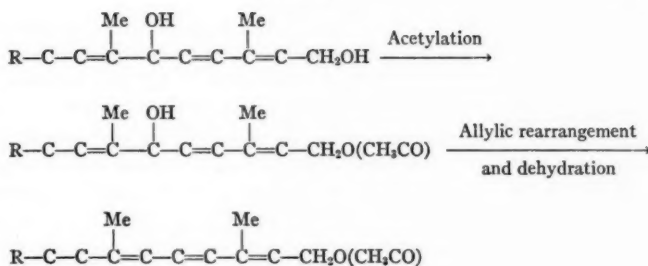
the ketone $\text{X}-\text{C}(\text{Me})=\text{O}$ is treated according to the following scheme:



The aldehyde was characterized as its crystallized semicarbazone (m.p. 207° – 209° with decomposition) and showed an absorption maximum ($\log E=4.87$) at $375.0 \text{ m}\mu$. This corresponds with the absorption spectrum of vitamin A aldehyde oxime obtained by Hunter & Hawkins (3) from natural sources. The aldehyde was reduced by means of aluminum isopropoxide and isopropyl alcohol, and after chromatography an oily fraction containing about 35 per cent vitamin A alcohol was demonstrated by bioassay, spectrophotometry, and conversion to anhydro vitamin A. These authors have also prepared β -ionylidene acetaldehyde [first described by Kuhn & Morris (4)] from β -ionone by a series of reactions similar to those outlined above. From this compound they proposed to repeat the Kuhn-Morris synthesis of vitamin A.

Isler *et al.* (5) have described a synthesis of vitamin A substantially similar to the one they reported (6) for vitamin A methyl ether, viz.:²





Vitamin A Acetate

Comparison of the synthetic esters with those derived from natural vitamin A, including mixed melting points, ultraviolet and infrared absorption spectra, Debye-Scherrer diagram, and biological activity, established that true vitamin A had been prepared.

In 1947 Cawley *et al.* (7) reported a physical and chemical comparison of natural and synthetic vitamin A, the latter having been obtained by an undisclosed method claimed to be different from those described in the literature. Clough *et al.* (8) have described the isolation of kitol by molecular distillation of the unsaponifiable extract of whale liver oil; it is also present in shark and dogfish liver oil. From the unsaponifiable extract of a crystalline di-*p*-phenylazobenzoate, pure kitol [m.p. 88°–90°, $E_{1\text{cm}}^{1\%}$ (290 mμ = 707] was prepared. Its analysis agreed with the formula $\text{C}_{40}\text{H}_{58}(\text{OH})_2$, double that of vitamin A.

Nomenclature.—Zechmeister (9) takes issue with certain aspects of the report of the Committee on Biochemical Nomenclature of the National Research Council on the Nomenclature of Carotenoid Pigments (10). Dyson (11) has illustrated the application of his system of abbreviation and enumeration of organic compounds to the carotenes. The reader is referred to the original source for details but an illustration may serve to incite interest. β-Carotene, according to the Dyson system, would have the cipher C18.1, 18(2AC6.1,1,3C.ZE).3,7,12,16C.1(2)17E.

Physiology.—Although the physiological mechanism for the conversion of carotenoids to vitamin A remains to be explained, it is certain that symmetrical fission is not the full answer. The biological activity of β-carotene for rats is only half that of vitamin A alcohol while the potency of other carotenoid precursors is half that

of β -carotene or even less. The problem is complicated by the large error of the bioassay and by variations in the efficiency of conversion to the active form of the vitamin. With (12) showed that β -oxycarotene, which is stored in the liver to only one-tenth the extent of α -carotene, has about the same biological activity, indicating that liver storage and growth promotion are not necessarily correlated phenomena. Cryptoxanthin, which is half as potent a β -carotene for the rat, is equally active for the hen. In this connection With points out that the only carotenoids deposited by the hen in its body fat and egg yolk are those containing OH in their ionone groups, cryptoxanthin being the only compound of this nature having vitamin A activity. These observations are especially significant from the standpoint of species variation and the applicability of a liver storage method of bioassay for products containing different forms of vitamin A and its precursors. It must be emphasized that evidence nevertheless exists for the *in vivo* fission of the central double bond in β -carotene. Gridgeman *et al.* (13) state that the fact that mutatochrome (14) has less than 47 per cent (and probably less than 39 per cent) of the provitamin A potency of β -carotene furnishes additional support for this concept.

Further light needs to be shed on the means by which marine species derive their preformed vitamin A, and on the purpose served by the enormous stores in the liver fats of various fish. Neilands (15) has described controlled feeding experiments demonstrating the capacity of *Gadus callarias* and *Homarus Americanus* to convert carotene to vitamin A albeit at a sluggish rate.

Using chromic oxide as a quantitative index of fecal excretion, Kreula (16) has studied the absorption of carotene from carrots in three human subjects. He found about 90 per cent excretion when finely grated carrots were consumed and about 30 to 50 per cent when carotene in oil was taken. [See also Callison & Orent-Keiles (59).]

Deuel *et al.* (17, 18) have reported evidence showing that the site of conversion of carotene to vitamin A in the rat is the intestinal wall; this transformation can occur *in vitro* by anaerobic incubation of excised intestine of vitamin A deficient rats after carotene dosage.

Considerable interest centers on the vitamin A and carotene content of colostrum and milk, especially in respect to the influence of supplemental feeding on milk and milk-fat production (19 to

27). Despite wide individual variations the vitamin A content of the colostrum of barn-fed heifers is high, especially after the first lactation, and diminishes at about the third milking (23). Pre-formed vitamin A in cow colostrum is practically entirely in esterified form (21). In lactating women under dietary control the vitamin A content was lower during the first two days postpartum, increased on the third or fourth day and then fell somewhat, whereas carotene was relatively higher on the first two days, reached a maximum on the third or fourth day and then decreased sharply (27).

The feeding of massive doses of vitamin A to lactating cows has been studied by several groups of investigators. Total milk and milk-fat production were not significantly affected by feeding "dry vitamin A" concentrate at the level of 1,250,000 units daily. Whereas the vitamin A levels of milk and blood serum, as well as the liver stores, were markedly increased by high vitamin A feeding, the carotenoid content was suppressed (25). The low efficiency of lactal secretion of dietary vitamin A, as well as the concomitant loss of carotenoid pigmentation of the milk fat, militate against vitamin A fortification of milk by this means. According to Hibbs & Krauss (22) and others, supplemental dosage of vitamin A at the rate of 10,000 to 25,000 units daily helps to overcome deficiency of vitamin A due to inadequate feeding of colostrum and whole milk, impaired absorption, or subsequent feeding of poor quality hays. However, neither the incidence nor the severity of scours in calves could be noticeably diminished through such dietary augmentation.

In connection with the foregoing observations on high vitamin A feeding, the paper of Sadhu & Brody is of interest (28). They found that large doses of vitamin A depressed the basal metabolism of rats and reduced the weight of their thyroids, although normal growth was not disturbed. The size of the thyroids of thiouracil-treated rats was also reduced and the metabolic stimulation of injected thyroxine inhibited by excessive vitamin A dosage. Further evidence for an antagonistic relationship between vitamin A and the thyroids may be seen in a study by Spanish workers (29), in which vitamin A deficiency developed in carotene-fed rats when receiving 0.01 per cent methylthiouracil in their drinking water. The symptoms disappeared when vitamin A in powdered thyroid was administered but continued despite the addition of more caro-

tene to the diet. These authors attribute their observations to inhibition of carotenase activity by thiouracil.

Distribution.—A collaborative investigation (30) of the seasonal variation in vitamin A and carotene content of retail butters showed minimal values during the late winter and early spring and maximal values during the summer and fall. The approximate limits were, for vitamin A, 0.45 to 0.95 mg. and, for carotene, 0.20 to 0.70 mg. per 100 gm. (equivalent to 8000 to 20,000 I.U. per pound). The "average" value of 15,640 I.U. for the six brands of butter tested was not weighted for the relative production of butter by months. The above range of vitamin A potency is somewhat lower than that reported by the United States Department of Agriculture in 1945 (31).

Space does not permit the enumeration of scores of reports, published from scattered sources throughout the world, on the vitamin A of fish liver oils and the carotenoid content of various plants or plant parts. Reference may be made, however, to the comprehensive study of South African fish products by the University of Capetown which has been appearing serially in *The Journal of the Society of Chemical Industry*.

Assay.—The United States Pharmacopoeial Revision Committee has adopted an oil solution of crystalline vitamin A acetate as a vitamin A standard to replace Reference Cod Liver Oil No. 3 which was conclusively shown to have lost potency. While it will undoubtedly prove to be more stable and uniform than its predecessor, the enhancement of the biological unit in the standard, as calibrated against pure β -carotene, has resulted in concomitant lowering of the apparent vitamin A values of vitamin A-bearing oils assayed against the old and new standards respectively. Because the U.S.P. announcement of the new standard stated that the conversion factor for calculating biological potency of an oil under assay, based on the E value for the nonsaponifiable fraction and the biological potency of 10,000 U.S.P. units per Gm. (of the vitamin A acetate standard) is 1894,

the latter has been considered to have official status as a conversion factor, despite the fact that the U.S.P. does not even describe an official spectrophotometric method of assay (except as a qualitative test under Cod Liver Oil). The situation is further confused by the knowledge that at least insofar as fish liver oils are concerned the factor 1894 overestimates the true biological activity of such oils.

The presence of irrelevant absorption in the ultraviolet absorption spectra of natural oils or extracts has stimulated interest in criteria for the evaluation and correction of absorption curves to minimize the effects of the interfering substances. The absorption curves of unsaponifiable extracts more nearly approach that of true vitamin A but deviations are apparent even after saponification. Oser *et al.* proposed certain limits beyond which vitamin A estimates, based on the extinction coefficient at 328 m μ , should be suspect (32).

An ingenious application of the geometry of symmetrical triangles has been proposed by Morton & Stubbs (33) as a means of correcting spectrophotometric curves in the region of the vitamin A absorption maximum to conform to that of the pure vitamin A present. The proportion of irrelevant absorption thus "removed" varied with the species of the oil, a few average values reported by these authors being: halibut 11.1, shark 16.1, dogfish 13.3, hake 10.6 per cent, the weighted means for all oils examined being 14 per cent.

Chromatographic separation of vitamin A from unsaponifiable extracts of low potency material, e.g., margarine, by means of flordin XXS (60 to 80 mesh), constitutes the basis for the determination of vitamin A described by Awapara *et al.* (34). The fact that this is a "difference method" and that the specificity of flordin as a vitamin A adsorbent is not fully established leaves room for doubt as to the accuracy of this relatively simple procedure.

Interest in adaptation of the Carr-Price reaction to the estimation of vitamin A and other carotenoids continues. The color reaction of carotenoids with antimony trichloride is regarded by Meunier (35) as caused by wandering of the electric charges across the system of conjugated double bonds, the intensity of color being proportional to the square of the length of the conjugated system. This concept is discussed in relation to the molecular composition and configuration of the carotenoids [see also (37)].

By means of an adaptation of the flowing cell apparatus of Gibson & Taylor (36), Caldwell & Hughes (37) were able to study the absorption spectra of the unstable products of the reaction between antimony trichloride and carotenoids. Vitamin A, which has a chromogenic power ten- to twenty-fivefold greater than that of the common carotenoids in the region of its absorption maximum (620 m μ), is unique in possessing a single strong absorption band

which rapidly decreases on standing. More complex changes are shown by β -carotene, lutein, and zeaxanthin, whereas α -carotene, lycopene, and cryptoxanthin show simpler curves which gradually rise or fall with time.

Johnson & Baumann (38) question the advisability of applying corrections for carotene in the colorimetric determination of vitamin A in crude mixtures since, among other interfering agents, oxidation products of the carotenoids are present in variable proportions. They were able to separate these interfering substances from carotene by extraction with 80 per cent methanol or, preferably, by chromatography.

The presence in fish-liver oils and in extracts of feeds of substances which affect the rate, and hence the intensity, of the Carr-Price reaction emphasizes the advisability of using an internal standard (the "increment technique") in this method. In this connection the observation of Tastaldi (39) that shark liver oil contains variable and sometimes high concentrations of inhibitors of the antimony trichloride reaction is significant, as is also that of Chechan *et al.* (40) that molecular distillates of shark liver oil are more chromogenic than the original oils. It seems more likely that this can be attributed to the removal of inhibitors rather than the conversion of a precursor into vitamin A, as claimed by these authors.

Sobel & Werbin (41) have reported on the use of activated 1,3-dichloro-2-propanol in the colorimetric estimation of vitamin A in fish-liver oils. For those laboratories not equipped with direct reading photometers, the stability of the color offers some advantage over that produced with antimony trichloride but difficulties have been experienced in respect to the sensitivity of the test and the reproducibility of the "activated" reagent. A simplified method for the use of this reagent in estimating vitamin A in serum is given by Sobel & Snow (42). Improved sensitivity is gained in the Coleman spectrophotometer by using a 5 cm. long cuvette; adaptations of the procedure to other types of photometers are described.

Caldwell *et al.* (43) have quite appropriately called attention to the necessity of calibrating individual photometers for the determination of vitamin A because of marked differences in the photo-cell and optical systems. Such calibration curves should be checked at frequent intervals. Considerations such as these lend added emphasis to the warnings against the assumption of constant L

values for the blue reaction product of vitamin A with antimony trichloride.

An important desideratum of the vitamin A industry is improvement in the precision of bioassays, yet little progress has been effected in this direction. The adoption of a method as "official" sometimes tends to inhibit the investigation of radical departures from "standard" procedure which may take years for "adoption." Theories for the *in vivo* conversion of carotenoids to vitamin A demand a greater degree of precision in bioassays than is to be found in some of the published data.

The need for an adequate supply of tocopherol in the basal vitamin A-deficient ration has been repeatedly emphasized. That one cannot rely on "vegetable oil" (cf. U.S.P. vitamin A-deficient diet) as a source of this factor is shown by Lemley *et al.* (44) in growth and liver-storage experiments. Supplementary feeding of tocopherols (0.3 mg. daily) augmented the growth response when olive or cottonseed oil was the fat component of the diet, but not when corn oil was used. This effect was apparent at low levels of vitamin A dosage but gradually diminished with increasing intake of vitamin A.

Cabell & Ellis (45) have investigated the efficiency, for optimum growth-promotion, of the U.S.P. vitamin A-deficient diet when supplemented with vitamin A, and have found that this basal diet lacks a factor which can be furnished by liver extract. This suggests the possibility that the missing factor may be the unidentified nutrient whose presence and amount in casein depends on its method of preparation [cf. (46)]. At any rate, Cabell & Ellis found that replacement of half the casein in the basal diet with pork muscle or especially with sardine meal (both ether-extracted) effected marked improvement in growth rate which was not further augmented by the addition of liver extract.

The liver-storage method of bioassay for vitamin A, originally proposed by Guggenheim & Koch (47), has been adapted by Foy & Morgareidge (48). Groups of weanling rats are fed the vitamin A-free ration and after six days depletion receive two successive daily doses of one hundred and fifty to three hundred units of vitamin A as the standard or unknown. On the fourth day after the initial dose the livers are removed, composited in groups of four and analyzed for vitamin A by the Gallup & Hoefer (49) adaptation of the antimony trichloride reaction. These authors stress the im-

portance of the oil used for dilution of the assay dose, as well as in the diet, since it has a marked influence on absorption and storage of vitamin A; this is particularly significant in assaying low potency oils (below ten thousand I.U. per gm.) because of the disproportionate ratios of assay and diluting oils at the two dosage levels employed. The inhibitory effect of sardine oil on the utilization (storage) of vitamin A could be partially counteracted by the addition of α -tocopherol.

• That caution must be exercised in applying a liver-storage assay to mixtures of carotenoids and their derivatives, or even to preformed vitamin A from various sources, is indicated by the work of several investigators. With's comparison (12) of β -oxy-carotene and α -carotene suggests that growth stimulation and liver storage may not always be parallel phenomena; Kemmerer *et al.* (50) have shown that xanthophylls and chlorophylls decreased utilization of carotene for liver storage about 20 per cent; Lemley *et al.* (51) compared liver storage of vitamin A after administering equal unit levels in different preparations, e.g., cod and halibut liver oils, saponified concentrate, acetate, and distillate. Storage was uniform in all cases except cod liver oil, different lots of which produced variable liver reserves. These authors (51) found the oral route of administration to be most effective for liver storage.

Assays for carotenes are based on a combination of chromatography and spectrophotometry. Among the numerous adsorbents that have been proposed, some of the English workers have a preference for bone meal (52, 53) whereas American workers prefer activated magnesia (54, 55). A mixture of acetone and hexane, e.g., 40 per cent (by volume) acetone in Skellysolve B (b.p. 65° to 67°), has been found to extract carotenoids from dried grasses more effectively than petroleum ether alone. Zscheile & Whitmore (55) reported the presence of neo- β -carotene as well as all-*trans*- β -carotene in fresh alfalfa, whereas in the dehydrated product isomers of the neo-U-, V-, and W β -carotene group were also found. These authors stress the view that

Different plants, plant parts, or products made from plants require individual treatment and study, as extraction methods, identity of pigments, and interpretation of both chromatographic columns (or other fractionation procedures) and characteristic absorption curves cannot be carried over arbitrarily from one material to another.

Continuing their series of papers on stereochemical configura-

tion and provitamin A activity, Deuel *et al.* (56) have reported the following relative potencies (β -carotene = 100) of cottonseed oil solutions of certain γ -isomers: all-*trans*- γ -carotene, 26; neo- γ -carotene-P, 19; mixed stereoisomers (with decreased absorbability), 16. Estimations of the provitamin A activity of foods by chromatographic separation of β -carotene from "other provitamins" (the latter being assumed to have half the potency defined for β -carotene) are likely to lead to serious discrepancies. For one thing they fail to take into account the differences in digestibility, absorption, and intestinal stability of carotenoids as influenced by the state of the food, other constituents of the diet, etc. These possibilities are recognized by Ramasarma *et al.* (57) who nevertheless report such a method. Significant in this connection are the recent observations of Sherman (58) that in the presence of unsaturated fats carotene is destroyed in the gastrointestinal tract of vitamin A-deficient rats unless α -tocopherol is also fed. Carotene was found to be relatively more stable than vitamin A when fed to vitamin A-deficient rats receiving fresh lard and no tocopherol. Addition of xanthophyll to the diet decreased the gastrointestinal destruction of carotene, vitamin A, and its acetate.

Of further interest in relation to the estimation of biological potency from analytical data is the work of Callison & Orent-Keiles (59) on the availability of carotene. They found about three times as much "vitamin A" in carrots by chemical analysis (using 0.6 $\mu\text{g.}$ β - and 1.2 $\mu\text{g.}$ α -carotene as equivalent to 1 I.U. of vitamin A) as by the rat-growth assay. Dark adaptation was employed as the criterion for availability of carotene from different sources to human beings. Variations were found not only among the foods tested (cooked carrots, peas, and spinach) but among individual subjects in their ability to utilize the carotene in these vegetables relative to the utilization of carotene in oil.

VITAMIN D

General.—A new synthesis of 7-dehydrocholesterol has been reported by Buisman and co-workers (60) involving the reaction between cholesteryl acetate and *N*-bromosuccinimide under exposure to radiation from a mercury vapor lamp. Depending upon the hydrolytic procedure employed, 7-dehydrocholesterol could finally be isolated as the digitonide or acetate. A method is described for irradiation and separation of unchanged sterols and

tachysterol to yield a vitamin D₃-cholesterol complex containing 46 per cent of active vitamin. Under conditions for ultraviolet irradiation of 7-dehydrocholesterol described by Waddell & Woessner (61), destructive loss of provitamin and vitamin during irradiation and processing is claimed to be held down by the presence in the mixture of cholesterol.

The products of the activation of ergosterol by high frequency electronic discharge (Whittier process) have been found by Dasler & Bauer (62) to include calciferol, neoergosterol, and an unidentified, inactive sterol (Steroid B) having no ultraviolet absorption above 250 m μ . Less than a third of the total antirachitic activity was accounted for by the calciferol which could be isolated.

From metallic vitaminates, Milas (63) has prepared biologically active alkyl ethers of vitamin D. The methyl ether had a potency slightly under 7.5 million U.S.P. units per gm., about half that estimated from the extinction coefficient of the antimony trichloride absorption maximum (499 m μ).

The antirachitic potency of dihydrotachysterol (AT10) for chicks has been investigated by Motzok *et al.* (64). Assuming McChesney's estimate (65) of 80 U.S.P. units per mg. in the rat, these workers found dihydrotachysterol to be approximately 7.5 times as potent for chicks, a ratio almost twice that reported in some of the earlier studies. Essentially normal values for blood calcium and phosphorus were obtained at all dosage levels, in disagreement with McChesney.

When incorporated into a practical poultry ration and stored in cotton bags at room temperature, the vitamin D in sun-rendered cod-liver oil was found to be more stable than vitamin D₃ added to such oil (66). In the former case the potency dropped to 60 per cent of its original level in fifteen weeks and remained stable for two years thereafter, whereas vitamin D₃ deteriorated progressively to about 30 per cent of the original value. The authors postulate the presence in cod-liver oils of varying proportions of stable and labile forms of vitamin D. The possibility of higher natural antioxidant content in unfortified oil (relative to vitamin D content) was not considered.

The effect of single massive doses of vitamin D₂ and of chronic high dosage of various forms of vitamin D on dogs has been studied (67, 68). Of the dogs fed 10,000 units per kg. daily for eight to ten months, all showed evidence of toxicity. Growth was di-

minished, soft tissues were calcified, long bones were hypercalcified, teeth were small and had deformed roots, and pulp stones were prevalent. Serum calcium was high but not consistently so. Some of the dogs received high vitamin A dosage, which seemed to alleviate the hypervitaminosis D. Of eight dogs administered single doses of 314,000 to 530,000 units of vitamin D₂ per kg., three died within two weeks and a fourth was moribund at five weeks, all showing symptoms of polyuria, bloody diarrhea, excessive thirst, anorexia, and prostration. The serum calcium level was elevated for a period of six months. Calcification of soft tissues, especially the lungs, was seen in all animals but especially in those which succumbed or were sacrificed soon after dosage.

Vollmer & Oser (69) compared the utilization of single massive doses of vitamin D by rats when administered parenterally in persic oil, or persic oil-ether solution, and orally in the oil alone. More prolonged protection or greater curative response was observed when the parenteral route was used, probably because of incomplete absorption via the gastrointestinal tract.

Houet (70) found that a single dose of 15 mg. of vitamin D₂ or D₃ effected improvement in calcium and phosphorus metabolism of rachitic infants, no difference being observed in response to the two forms of the vitamin. The prophylactic requirement of vitamin D₃ in oil, for infants, was found to be 250 units. The ratio of fecal to urinary phosphorus served as an index of vitamin D deficiency. The difficulties of quantitative collection of excreta limit the usefulness of this criterion in infants. However, by means of this ratio in studies on human adults, the daily requirement of vitamin D was estimated to be 3 to 4 μ g. (150 to 200 units) (71).

The forms of vitamin D in irradiated yeast and in cod-liver oil were found to be equally effective for growing pigs, the recommended intake being 90 U.S.P. units per pound of ration (72).

Natural and synthetic forms of vitamins D₂ and D₃ were studied by Gutteridge & Novikoff (73) for their effect on shell strength of eggs. The synthetic vitamins were found to be more effective than the fish-liver and body oils. The vitamin was fed at the level of 540 A.O.A.C. units per pound of feed. Thyroprotein (0.1 gm. per lb.) also promoted shell strength but was without effect on hatchability and egg production.

Assay.—Despite the relatively short duration of the depletion and assay periods in the U.S.P. bioassay for vitamin D, better

growth on the Steenbock-Black rachitogenic diet has long been recognized as a desideratum. Past efforts to improve the diet by additions of preformed vitamin A and vitamin B complex have not resulted in any significant improvement in the vitamin D response. A step forward seems to have been made by Francis (74) who compared the essential amino acid content of this diet (calculated from published data for corn and wheat gluten) with Rose's estimates of the requirements for rats and found a deficit of lysine. The addition of 0.5 per cent of this amino acid resulted in an improved rate of growth (22.5 gm. gain in sixteen days *vs.* 14.9 gm. for the controls) and earlier widening of the rachitic metaphysis. Blood fibrin (8.8 per cent lysine) also effected better growth but the appearance of the decalcified zone was less satisfactory. The advantage of lysine supplementation has been confirmed in this reviewer's laboratory and elsewhere (unpublished data).

A recently published note (75) recalls the experience of early workers in the field of vitamin D assay. Does fed a diet containing vitamin D-fortified dried milk for a four-month period produced rickets-resistant litters during the ensuing four months (when the milk was not fortified).

Statistical analysis of the extensive collaborative chick assays, conducted under the auspices of the Animal Vitamin Research Council (now the Animal Nutrition Research Council), have failed to reveal any material improvement in precision of the assay by modifying the A.O.A.C. basal ration to conform to more recent knowledge of the chick's requirements (76). The effect of variations in the calcium and phosphorus intake on the body weight and bone ash of chicks has been investigated by Migicovsky & Emslie (77). High and low calcium diets were prepared by appropriate substitution of CaCO_3 and KH_2PO_4 for the $\text{Ca}_3(\text{PO}_4)_2$ in the A.O.A.C. ration. At a given level of phosphorus intake, the body weight and percentage of bone ash rose with increasing calcium intake up to a level beyond which both responses decreased. Increasing the calcium to phosphorus ratio at a given level of calcium intake had no effect on bone ash except at excessive calcium levels, when it decreased. The effect of vitamin D on body weight was greatest when the calcium to phosphorus ratio was low; when it was high, vitamin D actually depressed growth. Continuing these studies the authors (78) found no difference in the slope of the dose:response curves whether the chicks received the A.O.A.C.

diet or the high- and low-calcium modifications. Comparison was based on three criteria of response (tibia ash, toe ash, and the tarsal-metatarsal distance), the relative activities of the diets being expressed in terms of the rachitogenic index, $1/\text{antilog } M$, where M is the horizontal distance between two dose:response curves.

The suggestion of a supplementary relationship between manganese and vitamin D in the diets of hens and pullets is contained in the work of Couch *et al.* (79). Egg production was better when hens received 24 mg. of manganese and the suboptimum level of thirty-eight units of vitamin D per 100 gm. of ration, than when they received 4 mg. of manganese and twice as much vitamin D, or approximately the minimum requirement.

Further evidence has been assembled in support of toe ash as the criterion of response in the chick assay rather than the more laborious, but no more reliable, bone-ash method (80, 81). Campbell & Emslie (82) have made a comparative study of the A.O.A.C. and British Standards Institute chick-assay diets and have statistically established the advantage of the one-week depletion period specified in the B.S.I. method. The A.O.A.C. diet, as judged by roentgenographic measurement, produced a somewhat greater degree of rickets. The procedure, employing a one-week depletion period followed by a three-week curative period, resulted in 35 to 40 per cent greater precision than the three-week preventive method of the A.O.A.C. This is equivalent to the precision that could be gained by doubling the number of chicks used in an A.O.A.C. assay. Unfortunately, these studies were based on the use of one diet for the preventive assays and another for the curative. However, the practice of placing "day-old" chicks on a stock or depletion diet for a week prior to commencing the assay period is not uncommon and merits wider critical consideration with a view toward possible official adoption of such a procedure.

In an effort to explain some of the variations reported in the requirement of turkey poults for vitamin D, Singen *et al.* (83) have investigated further the relationship between the source of the vitamin and the source of the phosphorus contained in the diet. They found that irradiated 7-dehydrocholesterol was more effective than cod-liver oil in promoting calcification in poults when the diet was high in phytin (cereal) phosphorus and low in noncereal (i.e., bone-meal) phosphorus. If the noncereal phosphorus were increased the difference in relative efficacy of the two forms of vita-

min D decreased. It would appear from these observations that vitamin D₃ may be involved in the utilization of both phytin and nonphytin phosphorus whereas part (if not all) of the vitamin D in cod-liver oil is effective only for nonphytin phosphorus. Increasing the level of vitamin D from 60 to 480 units per 100 gm. was less effective for calcification than an increase of 0.11 per cent in non-cereal phosphorus. A pertinent study by Hoff-Jørgensen (84) suggests that dried yeast possesses a strong inhibitory effect on phytase activity which, if manifested *in vivo*, may account for its previously observed rachitogenic effect.

Now that the U. S. Pharmacopoeia has dropped cod-liver oil as the standard for vitamin A and adopted crystalline vitamin A acetate, similar action is awaited in respect to the vitamin D standard. It would appear more likely that a new U.S.P. standard will be crystalline vitamin D₃ rather than calciferol (the International Standard) since there is an advantage in continuing the use of one standard for both rat and chick assays. Arnold (85) has assayed eight samples of commercial calciferol against the U.S.P. reference cod-liver oil and found an average potency of 49.7 ± 2.1 units per μg . Inasmuch as the activity of calciferol (by definition of the International Standard) is 40 units per μg ., these results are interpreted to indicate deterioration of the U.S.P. standard.

In 1939 collaborative work in England seemed to establish that crystalline vitamin D₃ and calciferol were equipotent for the rat. The purity of the former is questioned by Waddell & Kennedy (86) who report the results of several series of assays of crystalline D₃ prepared in the Winthrop and duPont laboratories. The results of these assays, all of which were conducted against the U.S.P. Reference Cod-Liver Oil No. 2, are tabulated below:

Sample of Cryst. D ₃	Number of Assays	Laboratories	Potency Found
			<i>A.O.A.C. units/μg.</i>
Winthrop	7	AVRC*	54.7 ± 2.82
Winthrop	16	duPont	52.7 ± 2.66
duPont	11	duPont	58.4 ± 2.75

* Animal Vitamin Research Council, seven collaborating laboratories.

(It is interesting to note that the last value recorded above was

The relative effectiveness of pure α -, β - and γ -tocopherols as antioxidants for vitamin A in fish-liver oils was reported to be enhanced when lecithin was added (91).

Hove & Harris (92) found that natural α -tocopherol was several times more active than the β - or γ -compounds in curing the creatinuria associated with muscular dystrophy in rabbits. Synthetic *dl*- γ -tocopherol had 30 per cent of the antidystrophic potency of natural *d*- γ -tocopherol. Intramuscular injection of *d*- α -tocopheryl phosphate effected a slower but more prolonged cure of dystrophy. The deficiency symptoms in rats on a vitamin E-low diet containing 5 per cent protein (crude casein) were prevented either by α -tocopherol or by increasing the protein level (93). The possibility that the low casein diet or some amino acid deficit may increase the requirement for vitamin E is under investigation. Tryptophane, valine, arginine, or lysine does not appear to be involved.

Harris *et al.* (94) reported that the ulcer-like lesion which develops in the stomachs of rats during recovery from deficiencies of vitamin A, vitamin B₆, or essential fatty acids could be cured by oral administration of α - or γ -tocopherol, but not by injections of α -tocopheryl phosphate. Possible explanations of the etiology of this syndrome were advanced.

Observations on young monkeys maintained for several years on a vitamin E-deficient diet have been made by Mason & Telford (95). Although reproductive functions were not studied because of the sexual immaturity of the animals, other symptoms characteristic of vitamin E deficiency in laboratory animals were noted, including muscular dystrophy and extensive deposition in the tissues of an acid-fast pigment. With respect to the latter a recent report (96) describes the attempt to separate the fraction in cod-liver oil which induces this pigmentation in vitamin E-deficient rats when the oil is fed at a 20 per cent level. Evidence indicates that the fatty acid fraction is involved. Meunier *et al.* (97) have observed that the addition of 5 per cent cod-liver oil to the diet of young rabbits greatly increased their vitamin E requirement.

The interrelationships of dietary fat and the tocopherols have been reviewed by Mason & Filer (98). Emphasis is placed on the antioxidant role of the tocopherols not only in the diet before ingestion but during digestion, mobilization, metabolism, and storage of unsaturated fats.

Whether the classic histopathologic manifestations of vitamin E deficiency (feta)

death, testis degeneration, muscle dystrophy), which it has been pointed out can occur in the absence of dietary fat, but are accentuated by ingestion of unsaturated fatty acids, are due to unstable states arising in tissue lipids synthesized by the animal from other sources, or are due to disturbance of some specific cellular enzyme system in which tocopherols participate by virtue of properties other than those of antioxidants, remains for future research to determine.

Ames (99) explains the recent failure of Basinski & Hummel (100) to corroborate the earlier report from their own laboratory that increased succinic dehydrogenase activity in dystrophic muscle could be depressed to normal *in vitro* by addition of α -tocopheryl phosphate. Ames postulates that inhibition by *d*- α -tocopheryl phosphate is based upon its capacity to remove calcium ions, resulting in lack of activation of diphosphopyridine nucleotidase. The persistence of diphosphopyridine nucleotide results in inhibition of succinic dehydrogenase by the oxaloacetate formed from the diphosphopyridine nucleotide-linked malate system. He attributes the high oxygen uptake observed in dystrophic-muscle slices to the ability of α -tocopheryl phosphate to combine with and remove the calcium from the homogenate of the tissue which is characteristically high in calcium. It is not clear however how this mechanism explains Basinski & Hummel's observation that tocopheryl succinate was equally effective as the phosphate in inhibiting succinic dehydrogenase of normal and dystrophic muscle *in vitro*.

The availability of a sensitive and precise method for the estimation of tocopherols in body fluids and tissues would help to remove much of the current speculation as to their mode of action. A step in this direction has been made by Quaife and co-workers (101) who have adapted the Emmerie-Engel reaction for tocopherol to blood plasma (102) and milk (101). In this connection reference may be made to the observation by Emmerie (103) that cholesterol or ergosterol does not interfere with the ferric chloride-bipyridyl reaction unless they contain impurities which can be removed by filtration through floridin XS. The substitution of glacial acetic acid for alcohol as a solvent in this reaction is not recommended.

Kaunitz & Beaver (104) applied their method to the determination of tocopherols in muscle tissue, claiming agreement with bioassays to within ± 10 per cent. Faaborg-Andersen (105) found no difference in tocopherol content in the blood of normal, preg-

nant, and aborting women, the range being 2 to 17 $\mu\text{g.}$ per c.mm. The method employed was that of Glavind *et al.* (106). Lundberg *et al.* (107) found by oxygen-absorption measurements that α - and β -tocopherol were deposited to a greater extent than the γ -form in the abdominal and ham fat (but not in skin fat) of vitamin E-deficient rats. No significant differences were observed between the natural and synthetic forms of these tocopherols either in antioxidant properties or in amounts deposited in fatty tissue.

It has been reported by Martin (108) that the fat metabolism in vitamin E deficiency is entirely reversed in the castrate rat.

An increase in the average fat content of milk from 3.47 to 4.42 per cent and in total ("4 per cent") milk production from 35.01 to 42.59 lb. per day has been claimed by Harris *et al.* (109) to result from feeding cows a daily supplement of one gm. of "mixed tocopherols." The efficiency of transfer of vitamin A and carotene into the milk was not enhanced and little, if any, difference was obtained in the milk or blood tocopherol levels compared with the unsupplemented controls. Insufficient data were presented concerning the prior nutritional history of these cows to establish whether the effects observed resulted from improvement of their nutritional status or from specific stimulation of the lactation process.

ASCORBIC ACID

Metabolism.—The relation between intake and blood and urine levels of ascorbic acid under conditions of physiological stress has occupied the attention of several groups of investigators. A series of studies from Japan (110) reported blood levels of 0.9 to 1.3 mg. per 100 ml. on normal diets, the minimum daily requirement being regarded as close to 30 mg. while 80 mg. was above the saturation point. Direct correlation was observed between ascorbic acid dosage and blood levels (111).

Using their microtechnique for the estimation of ascorbic acid, Lowry and associates (112) determined the effect on serum and white blood cell concentrations in subjects receiving standardized dietary ascorbic acid levels over an eight-month period. At 8 or 23 mg. of ascorbic acid per day, the concentration in the white blood cells averaged about 12 mg. per 100 ml. as against twice this level for those receiving 78 mg. daily; in the serum the values averaged 0.2 mg. per 100 ml. at 8 and 23 mg. levels of intake and 0.8 mg. per 100 ml. when the diet furnished 78 mg. daily. In a

study on forty-one female college students Dodds & MacLeod (113) estimated plasma ascorbic acid after the administration of 0, 50, 75, and 100 mg. doses of the vitamin as supplements to a diet furnishing 7 mg.; the values averaged, respectively, 0.48, 0.72, 0.93, and 1.05 mg. per 100 ml. but the extent of overlapping of the ranges was such as to preclude narrow definition of the ascorbic acid intake of a population from plasma levels.

Klosterman *et al.* (114) have modified their earlier method for estimating the renal threshold for ascorbic acid. Hourly urinary excretion was determined for plasma values 0.1 mg. per 100 ml. above and below the apparent threshold for each subject as estimated from scatter diagrams. The renal threshold for twelve normal adults was found to be 1.0 to 1.3 mg. per 100 ml. Hauck (115) observed no unusual variability in urinary or plasma ascorbic acid values associated with the menstrual cycle. Metabolic studies in nursing mothers by Munks *et al.* (116) have shown no consistent relation between the urinary excretion and the ascorbic acid concentration or output in the milk. The results were quite variable but in general the amounts of ascorbic acid in milk paralleled the milk volume. The daily allowance during lactation of 150 mg. of ascorbic acid, recommended by the Food and Nutrition Board of the National Research Council, was found to suffice where prenatal nutrition was satisfactory (116).

In this connection, attention may be drawn to the findings of Sheahan (117) on the ascorbic acid content of the blood serum of cows, bulls, sheep, and pigs. The level dropped during the winter and rose in the spring, but no correlation was observed with the ascorbic acid content of the fluids of the reproductive organs; nor could a relation be established between ascorbic acid levels and reproductive dysfunction in the bulls. Storvick *et al.* (118) found that N.R.C. recommendations for ascorbic acid in adolescents resulted in satisfactory fasting plasma values (more than 0.6 mg. per 100 ml.) although they fell below the levels attained in saturation tests.

The wide variations in the same and in different individuals in the ascorbic acid content of fasting urine or plasma would appear to cast considerable doubt on the validity of these determinations as indices of individual nutritional status with respect to this vitamin. Whether, and to what extent, dietary flavones contribute to this situation remains for future investigation.

While therapeutic applications of ascorbic acid are not within the scope of this survey, mention should be made of the work of Wolfer *et al.* (119) on wound healing in human subjects under conditions of prolonged ascorbic acid depletion. The lack of collagen and reticulum observed in such wounds was sufficient to reduce the tensile strength by 50 per cent and delay healing by as much as two weeks. These observations stress the need for maintaining high preoperative levels of ascorbic acid in tissues to promote resistance to infection and to favor the healing process.

In a continuation of studies on the role of ascorbic acid in the metabolism of the melanin precursors, Sealock & Lan (120) have demonstrated that kidney slices from scorbutic guinea pigs were unable to oxidize 3,4-dihydroxyphenylalanine. The effect was reversible upon addition of ascorbic acid. Similar results were observed *in vivo*. Liver slices from the same animals were much less active in this regard and, under the experimental conditions used, amino acid oxidation in this tissue seemed to be independent of ascorbic acid levels. It has been suggested that anaerobic decarboxylation of amino acids without subsequent oxidation of the resulting pressor amine would be expected to induce hypertension. Thus the failure of amino acid catabolism in ascorbic acid deficiency may have an important bearing on the problem of hypertension.

Dugal & Thérien (121) reported an interesting study of the effect of prolonged adaptation to a cold environment on the ascorbic acid metabolism of rats and guinea pigs. The kidneys, and to a lesser extent the liver and testes, of rats adapted to cold (as indicated by their growth) showed a marked rise in ascorbic acid content, but in rats not adapted to this environment the levels decreased. Resistance and cold adaptation in guinea pigs were directly related to the amount of supplementary ascorbic acid fed daily. Adapted guinea pigs maintained or gained weight and retained more ascorbic acid in their organs, including the adrenals, than nonadapted animals.

Assay.—In view of the question of specificity that beclouds most chemical methods for the assay of ascorbic acid, and the need for greater precision and simplicity of the bioassay, the advent of a new biological method should be greeted with some interest. Crampton (122) has developed a procedure based on his observation that the length of the odontoblast cells in guinea pigs is correlated with ascorbic acid intake, varying from 30 to 70 μ in length

in the dosage range of 0.5 to 2.0 mg. of ascorbic acid. The precision of the proposed method was about two to four times as good as that of the growth method in a series of assays of fruit juices. However, the fact that, in addition to the six week assay period, the method requires the dissection of the teeth, decalcification, sectioning, embedding, staining, and examination, which consume another ten days, does not augur well for its wide acceptance.

In their attempt to arrive at a nutritionally complete basal diet for the assay of ascorbic acid, Crampton & Bell (123) investigated the reproductive performance of guinea pigs on their "Macdonald No. 5" diet, as influenced by the addition of natural *vs.* synthetic sources of ascorbic acid and of "greenfeeds" (fresh grass or cabbage). Fresh orange juice, lemon juice (decitrated), and even a synthetic orange juice, induced better reproductive response and protection against hemorrhage than aqueous ascorbic acid. The improved response resulting from the further addition of fresh "greenfeed" suggests that the basal diet was not complete. Hence any inference from this work that natural ascorbic acid is superior to the synthetic compound would not be warranted.

The necessity in chemical assays for ascorbic acid to avoid or correct for the interference of nonspecific reducing substances is emphasized by Miller (124). She analyzed various fresh and preserved fruits and vegetables by the indophenol-xylene extraction method of Robinson & Stotz (125), with and without their formaldehyde correction for nonspecific reductones, and by the direct dye-titration method where color interference was not too great. As might be expected from earlier observations, fresh fruits or vegetables evidenced no reductone interference but all preserved, canned, concentrated, or dehydrated foods did to degrees ranging from 11 to 70 per cent. During prolonged storage under refrigeration, the nonspecific reduction increased more rapidly in foods of low ascorbic acid content than in those of high content. Hydrogen sulfide treatment resulted in much larger increases in dye-reducing values of the preserved foods in contrast with the fresh, although in the case of fresh cranberry sauce and certain citrus concentrates, this increase was largely eliminated by the formaldehyde correction. As this author significantly points out when unmodified dye-reduction methods are employed, "apparent" ascorbic acid content or stability observed in stored, processed foods may be definitely misleading.

The usefulness of oxalic acid as an extractant for ascorbic acid

was pointed out by Ponting (126) and its advantages and limitations have been discussed by Wokes (127). Heulin & Stephens (128) demonstrated the strong inhibitory effect of 0.01 *M* oxalate on copper-catalyzed oxidation of ascorbic acid; the influence on iron-catalyzed oxidation was considerably less. The effects of strong and moderate acidity on these oxidation reactions are discussed and methods suggested for determining ascorbic acid in the presence of ferrous iron.

Bolomey & Kemmerer (129), on the basis of spectrophotometric evidence, have qualified their previous recommendation that glacial acetic acid be substituted for 85 per cent sulfuric acid in Roe's dinitrophenylhydrazine method for dehydroascorbic acid. Though acetic acid avoids charring, sulfuric acid provides better differentiation of the absorption maxima and is to be preferred when an ordinary colorimeter is used instead of a spectrophotometer in which the spectral limits can be closely defined. [It may be noted that Bessey *et al.* (130), in their microadaptation of the Roe method, use 65 per cent sulfuric acid.] In either case, the necessity for a blank correction is pointed out. In view of previous efforts to improve the specificity of Roe's method by taking advantage of the difference in reaction rates of dehydroascorbic acid and reductones with dinitrophenylhydrazine, the experiment of Bolomey & Kenimerer showing that the reductones themselves react at different rates may be significant. However, the modifications proposed by these authors are strongly opposed by Mills & Roe (131) on the ground that the essential reagent thiourea had been omitted. The presence of this reducing agent is claimed to be necessary to control the rate of the coupling reaction and thus make it more specific for ascorbic acid. Under these conditions, and using sulfuric acid as originally proposed, a blank correction curve based on the differences in values for filtrates before and after oxidation is not necessary because such differences are not observed. It is further claimed by Mills & Roe that greater specificity is obtained in the analysis of plant and animal tissues when a 540 $m\mu$ filter is used rather than a 520 $m\mu$ filter. The latter may be used when interfering substances are not present in significant amounts, as in blood.

Papers too numerous to discuss here have appeared from all quarters of the globe on the distribution and retention of ascorbic acid in plants and plant parts, reflecting the widespread efforts to

meet nutritional requirements from local sources. Among the more recent reports may be cited those of L'vov (132) on the ascorbic acid content of different varieties of wild rose hips (ranging from 2.75 to 8.75 per cent); of Tuba *et al.* (133) on the distribution of "true ascorbic acid" and "non-vitamin C reductants" in green walnuts; of Baker & Parkinson (134) on the loss of ascorbic acid in tomatoes after picking; and of Branion *et al.* (135) showing a loss of 60 to 70 per cent in potatoes upon storage for six to seven months. Petersen & Schönheyder (136) found that the ascorbic acid content of dehydrated kale, white cabbage, and spinach diminishes according to a first order reaction when stored at constant temperature and humidity. The time of half decomposition of ascorbic acid was inversely related to moisture content, in contrast with these authors' observations on the stability of carotene in carrots which showed a peak at a water content of 18 per cent.

A new process for the dehydration of orange juice (137) involves concentration by vacuum diffusion at 55°F. to 50 to 60 per cent solids followed by addition of fresh juice (to 44 per cent solids) to restore volatiles and finally drying to 1.5 per cent moisture at room temperature and under only a few microns pressure.

The report by Crampton & Burton (138) that fruit juices contain a factor which significantly enhances the biological potency of the chemically determinable ascorbic acid prompted Melnick *et al.* (139) to investigate this possibility by means of their human bioassay technique (140). Whereas no greater availability was observed for ascorbic acid when added to apple juice, the vitamin was more stable than in aqueous solution.

VITAMIN P

Progress in the elucidation of the specific role of the antihemorrhagic flavone glycosides ("vitamin P") has been slow, owing in no small degree to the difficulties of quantitative evaluation and of adequate differentiation from the behavior of ascorbic acid. Much of the clinical evidence has been inconclusive because of inadequate controls.

The production of the deficiency syndrome in guinea pigs has been described by Parrot, Gabe & Cotereau (141) who also studied histological changes in the thyroid and adrenals. The marked capillary fragility observed could not be reversed by ascorbic acid; similarly with respect to adrenal pathology despite its resemblance

to that seen in scurvy. The hyperactive appearance of the thyroid could be restored to normal by simultaneously administering ascorbic acid and vitamin P but not by either alone. If anything, only a slight decrease in thyroid activity could be induced by these investigators (142) by the daily injection of mixed epimers of catechin to normal guinea pigs or rats.

To meet the need for a satisfactory quantitative bioassay, Hughes & Parkes (143) propose a modification of the method of Bourne (144). Their criterion is the proportion of guinea pigs (in groups of fifteen or twenty) showing a rise of 150 mm. in capillary resistance to the production of hematoma under applied suction. These authors have determined the activity of extracts of citrus peels and of a number of synthetic compounds, particularly chalcone derivatives.

Following the discovery by Couch *et al.* (145) that the green buckwheat plant contained eight to twelve times as much of the active flavonol glycoside, rutin, as flue-cured tobacco and, having demonstrated its availability in commercial quantities (146, 147), extensive clinical studies have been undertaken. A summarized statement prepared by Griffith reported (148) that in dosages seldom exceeding 60 mg. per day, rutin has produced increased capillary resistance in 21 per cent of 1219 cases involving hemorrhagic conditions, as measured by the Göthlin test (subcutaneous petechiae). Rutin has also been claimed to be of value in counteracting the increased capillary fragility resulting from various drugs like thiocyanates, salicylates, and arsenicals. It is significant, however, that an adequate intake of ascorbic acid is reported to improve the effectiveness of rutin.

Studies have been reported by Haley *et al.* (149) on the relative activity of topically applied vitamin P-like compounds on the microscopically visible vasomotor response of capillaries in a preparation of the mesoappendix mesenteric from the rat. Catechins, including *d*-catechins, epimers of *d*-catechin, and especially *l*-epicatechin, showed high vasoconstrictor activity whereas rutin, hesperidin, and their sodium acid succinates, as well as methylated hesperidin chalcone, esculin, and adrenochrome, were inactive. Slight vasoconstriction was observed with the sodium acid phthalates of rutin and hesperidin.

A satisfactory explanation of the mechanism of vitamin P activity remains to be offered. On the basis that it retards neuro-

muscular symptoms in guinea pigs and prevents transient symptoms in rats on ascorbic acid-free diets, Lecoq *et al.* (150) suggest that vitamin P exerts a sparing action on ascorbic acid. Parrot & Cotereau (151) demonstrated *in vitro* that mixed epicatechins inhibited the oxidation of ascorbic acid and of epinephrine. Oxidation of ascorbic acid was accelerated by "antivitamin P" extracted from *Brassica napus*, var. *esculenta*. That the flavonols function as inhibitors of the oxidation of epinephrine was postulated by Lavollay (152). He suggested, in fact, that "true vitamin P" was either epinephrine itself or an oxidation product. With Neumann (153) he showed that the flavonols, rutin, and quercitrin retarded oxidation of epinephrine in the blood thus prolonging its action. Preliminary injection of quercitrin prevented peptone shock. The oxidation of epinephrine in hydrogen peroxide-peroxidase systems was accelerated by the flavonols but not by the flavanones (e.g., hesperidin).

Noting the observation that hyaluronidase tends to increase capillary fragility (154) Beiler & Martin (155) have recently investigated the possibility that vitamin P substances function through inhibition of hyaluronidase activity. They found that ascorbic acid and dicoumarol exerted an inhibitory effect whereas, of the other compounds tested, including hesperidin, its methyl chalcone, esculin, esculetin, its methyl derivative, and rutin, only the latter was active but in higher concentration. However, in combination with ascorbic acid these compounds showed a marked synergistic action, especially in the case of hesperidin methyl chalcone. Such potentiation did not occur when the latter was combined with dicoumarol. Neither sulfanilamide, salicylic acid (alone or with ascorbic acid), nor salicyluric acid showed an inhibitory effect on hyaluronidase activity.

Quercetin, quercitrin, hesperidin chalcone (but not hesperidin itself), and rutin have been shown by Richardson *et al.* (156) to be effective antioxidants for milk fat and lard. It is suggested that this activity is due to the grouping $-\text{CO}-\text{C}=\text{C}-$ in the pyrone ring or in the open chalcone.

In view of the multiplicity of natural compounds known to possess vitamin P activity, estimations of potency by chemical or physical methods are limited to relatively pure extracts of known composition. Bioassays for vitamin P based on prevention or cure of petechial hemorrhages in guinea pigs have not been uniformly

successful. In an excellent critical review on the value of capillary strength tests in diagnosing human deficiency of vitamins C and P, Munro *et al.* (157) stress the nonspecificity of this criterion as an index of dietary deficiency. In fact they regard the evidence for the essentiality of vitamin P in the diet of man as insufficient and inconclusive.

Of the various pharmacological properties of vitamin P, the one more recently adapted for assay purposes is its antioxidant action toward epinephrine. This can be measured by the prolonged inhibition of epinephrine-induced contraction of excised intestinal muscle of the guinea pig (158) which, according to De Eds (159), is more adaptable to quantitative measurement than observations on the intact animal. The antioxidant property toward epinephrine may also be measured by the Warburg technic. However, it must be recognized that this type of procedure is based on only one property of vitamin P which may vary in degree, as compared with other properties, depending upon the source of the activity. Another bioassay procedure, employed with some success by de Eds and associates, is based on the time required for intravenously injected trypan blue to escape from the capillaries at the site of a chloroform burn in rabbits.

A tentative spectrophotometric method for rutin in crude and purified preparations has been described by Porter *et al.* (160). The method is based on the absorption maximum at 362.5 μ and the density ratio $D_{375.0}/D_{362.5}$. An isolation procedure for the determination of hesperidin and eriodictin in natural material has been described by Pallares & Orozco (161). It is based on carbon dioxide precipitation of the two compounds from an alkaline alcoholic extract, estimating the former from its methoxy content and the latter by difference.

By way of special interest to gourmets, mention may be made of the pleasing report of Lavollay & Sevestre (162) that wine owes its "tonic" value in no small measure to its high content of vitamin P. This observation is based on experiments in which wine was administered orally and parenterally (*horribile dictu!*) to guinea pigs and found to increase capillary resistance.

VITAMIN B GROUP

Since certain papers deal with the "vitamin B-complex" or with groups of these vitamins, consideration will be given them under

this heading rather than in subsequent sections devoted to the individual factors. Webb & Loofbrourow (163) have compared the content of vitamin B factors in intercellular suspension fluids from normal and ultraviolet-damaged yeast cells with a view toward determining the role of these vitamins as "wound hormones," i.e., factors promoting the proliferation of damaged, but intact, cells. Higher concentrations of all the B factors were found in the fluid from damaged cells, the ratios ranging from 2.3-fold in the case of inositol to 91.5-fold for pantothenic acid. The possibility was considered of photochemical destruction in those instances where the ratios were low. In any case, the vitamin B factors were found to account for part, but not all, of the proliferation-promoting effect on the growth of damaged yeast cells. Whether the irradiation process caused release of bound vitamins does not appear to have been taken into account, according to the description of the assay procedures employed.

Several reports have appeared on the extensive study of the effects of restricted vitamin B complex and protein intake on young men, undertaken under the auspices of the United States Army (164 to 167). The vitamin content of feces was as high, or higher, on the restricted diet as on the normal diet, whereas urinary excretion dropped markedly, indicating that bacterial syntheses continued to take place and, to a large extent, in the lower bowel where absorption was slight. In the case of certain of these vitamins, where the requirements are known to be low, intestinal synthesis may occur to a nutritionally significant extent. The urinary excretion of pantothenic acid and biotin approximated and sometimes exceeded dietary intake suggesting either that absorption occurred through the large intestine or that these vitamins originated from an unidentified metabolic process in the tissues. During the period of restricted vitamin and protein intake (25 to 45 per cent of normal levels), the concentrations of folic acid, biotin, pantothenic acid, nicotinic acid, and tryptophane in the blood were not appreciably diminished.

Whereas both normal and vitamin B complex-deficient rats are able to inactivate estrone *in vivo*, Zondek & Finkelstein (168) have reported that brei prepared from the liver of deficient rats is incapable of effecting this destruction *in vitro*, in contrast to the usual finding in the case of normal liver brei.

Deficiencies of vitamins of the B group and their requirement

have been studied by Jukes *et al.* (169) for turkey poults, by Schaefer *et al.* (170) for foxes and by McLaren *et al.* (170a) for rainbow trout. Of special interest to investigators in poultry nutrition is the achievement by Jukes & Stokstad (171) of rearing chickens, from the time of hatching, on a purified diet in which all the known B vitamins (except inositol) were in synthetic form. Reproduction occurred although hatchability of the eggs was poor; however, some normal but small chicks were obtained. Mills *et al.* (172) have studied the effect of environmental temperature on the requirement of chicks for thiamine, pyridoxine, nicotinic acid, pteroylglutamic acid, and choline. No differences were observed except in the case of thiamine where the polyneuritic threshold level, on exposure to tropical heat, was found to be 3 mg. per kg. of diet as compared with 1 mg. at temperate coolness.

The content of thiamine, riboflavin, nicotinic acid, pantothenic acid, and reduced ascorbic acid in mare's colostrum and milk has been investigated by Pearson (173).

THIAMINE

With a view toward studying the mechanism of inhibition of the Chastek paralysis enzyme of fish tissues by compounds structurally related to thiamine, Livermore & Sealock (174) have synthesized a series of benzyl-(3)-thiazolium chloride analogues of the vitamin. The thiaminase activity of the muscle and viscera of a series of fresh and salt water animals was investigated by Neilands (175). The enzyme was found in most of the fresh water fish but in only one teleost and four invertebrates among the marine forms. Thiamine analyses of various species of fish from Lake Michigan, as influenced by storage and preparation, have been studied by Klocke *et al.* (176). Of these the lake herring and burbot appear to be highest in thiamine, averaging about 0.87 and 3.78 μ g. per gm. of fresh tissue, respectively.

The effect of thiamine deficiency on pyruvate oxidation in dogs has been investigated by Himwich & Himwich (177). They found a pyruvate cycle in the normal animal, as well as in the deficient, hepatic glucose being exchanged for muscular and intestinal pyruvate. The liver removed more pyruvate from the circulation in thiamine-deficient dogs than in normal animals. The only organ which added rather than removed pyruvate from the hyperpyruvemic blood of thiamine-deficient dogs with neurological symp-

toms was the heart (178). Since T-wave alterations were commonly observed in these animals, the authors suggest that thiamine deficiency be considered in patients showing unaccountable T-wave changes. Foà *et al.* (179) have observed that insulin catalyzes the phosphorylation of thiamine in dogs, thus suggesting the possibility of impaired thiamine metabolism in diabetes, despite the fact that substantial evidence indicates that this condition is not encountered in clinical diabetes. The role of thiamine and cocarboxylase in bioelectric phenomena and in the metabolism of acetylcholine in the nervous system has been the subject of other reports (180 to 183).

Cytological studies of the adrenal and thymus glands in rats deficient in thiamine, riboflavin, or pyridoxine have revealed stimulation followed by exhaustion of the zona fasciculata of the adrenal cortex and concomitant atrophy of the thymus, similar to that observed in pantothenic acid deficiency [Deane & Shaw (184)]. Neither riboflavin nor pyridoxine deficiency caused more than a transitory adrenal stimulation but lack of pyridoxine resulted in acute involution of the thymus. It is to be noted that the diet employed by these authors did not include a source of folic acid.

Further studies on the physiological availability to human subjects of thiamine in fresh and dried yeasts have been reported by Kingsley & Parsons (185). Not only was the thiamine in viable fresh or dried yeast unavailable for absorption, but actual reduction of urinary excretion of thiamine below the control levels was observed, indicating interference with the absorption of dietary thiamine by competition between the live yeast cells and the host. The more prolonged the period of ingestion of large doses (150 gm. per day) of fresh yeast, the greater was the delay in the return to basal levels of urinary excretion. This has been interpreted as indicating progressive depletion of the body stores of thiamine, although the possibility of continued intestinal propagation of yeast cells does not seem to have been excluded.

A more convenient method than the clearance test (186) for determining the status of thiamine nutrition is proposed by Papageorge & Lewis (187). It is based on the thiamine content of the urine collected during a one-hour fast following the morning emptying of the bladder and drinking of a glass of water. No dietary control is imposed during the preceding twenty-four hours but beginning with the fourteenth hour water only is permitted to

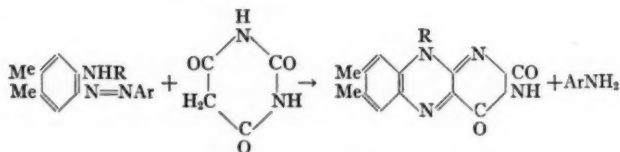
be ingested. Under these conditions the critical level of thiamine excretion during the fasting hour is 4 $\mu\text{g.}$; lower values suggest thiamine inadequacy. Of interest in this connection are the observations of Berryman *et al.* (188) that the thiamine or riboflavin depletion of the liver, heart, and kidney of rats on diets deficient in these vitamins was more accurately reflected by load-test response than by fasting urinary levels. The latter appeared to be governed by immediately preceding levels of intake of these vitamins.

Mickelsen *et al.* (189) have corroborated the linear relation between thiamine intake and excretion and emphasize the wide variations observed among different individuals. They suggest that pyrimin excretion, which is exponentially related to thiamine excretion, offers a better index of thiamine nutrition because individual differences are smaller and excretion continues to be demonstrable when the level of thiamine intake is so low as to cause its disappearance from the urine.

A variety of inorganic and organic compounds, particularly phenolic substances present in plant materials, have been demonstrated to interfere with the thiochrome reaction for thiamine determination (190, 190a).

RIBOFLAVIN

Synthesis.—Tishler and associates (191) at the Merck laboratories have reported a new synthesis of riboflavin and related compounds, involving the reaction between an appropriate *o*-aminoazo compound and barbituric acid.



When the Ar group was *p*-nitrophenyl and R was *D*-ribityl, the yield of riboflavin was 65.8 per cent. A similar tetraacetyl-*D*-ribitylamino compound was prepared from which riboflavin was obtained in even higher yield by catalytic deacetylation in methanol.

Among the more recent methods proposed for increasing the solubility of riboflavin (192, 193) an especially interesting one is

that of Frost (194) which employs aqueous nicotinamide solutions as the solvent medium. At pH 5 the solubility of riboflavin increases from 0.1 to 2.5 per cent as the nicotinamide concentration increases from 5 to 50 per cent.

Physiology.—The riboflavin requirement of fowl has been studied by various groups of investigators (195 to 199). The evidence indicates that the requirement of chicks for optimum growth, about 3 μ g. per 100 gm. of ration, is insufficient for breeding hens, which should have between 3.6 and 5.1 μ g. per 100 gm. of ration, or even higher, for maximum egg production and hatchability. Turkey poults require somewhat more riboflavin than chicks for optimum growth (195).

Among the factors which retard the development of tumors in rats receiving *p*-dimethylaminoazobenzene in a diet favoring early carcinogenesis is riboflavin, incorporated to the extent of 5 to 8 mg. per 100 gm. diet (containing 20 per cent casein). One per cent cysteine (or cystine) or 3 per cent liver extract has a similar inhibitory effect (200). Of interest in this connection is the observation (201) that carcinogenic azo dyes reduced the riboflavin content of the liver, the more so the greater their carcinogenic power. The riboflavin content of the kidney, heart, and skeletal muscles was not similarly affected.

Fenton & Cowgill (202) have found two inbred strains of mice, characterized by high and low incidence of spontaneous mammary tumors, to require for maximal growth 0.6 and 0.4 mg. of riboflavin, respectively, per 100 gm. of diet.

Assay.—Although the microbiological assay for riboflavin remains the method of choice from the standpoint of specificity, the fluorometric procedure has advantages which make it more readily adaptable to clinical use. Scott *et al.* (203) and Slater & Morell (204) have reported improvements which, they claim, lead to closer agreement with microbiological assays. The use of higher concentrations of potassium permanganate is recommended to remove interfering pigments although excessive amounts tend to destroy riboflavin. However, both groups of investigators employ an internal riboflavin standard to correct for quenching of the fluorescence. Slater & Morell also introduce a step involving photolytic destruction of riboflavin as an added check on the specificity of the method. These authors (205) have compared their own procedure with other fluorometric methods for riboflavin in urine.

They found high values by all methods employing the hydrosulfite blank, whereas Najjar's methods (206) gave low values for urines of high riboflavin concentration and vice versa. Loy (206a) has combined the best features of several previously published fluorometric methods for riboflavin in the attempt to adapt a more generally applicable procedure.

NICOTINIC ACID

During the past year a series of patents has been issued for the synthesis of nicotinic acid and its derivatives. One (207) describes the preparation of nicotinamide by reacting nicotinic acid with an excess of dry ammonia at 230° to 235°, the resultant water being removed by the excess ammonia. A synthesis of nicotinic acid is described (208) based on demethylation of trigonelline with HBr and NH_4Br in the presence of zinc or gold salts at 350°. Another synthesis (209) of nicotinic acid involves the oxidation of 3-picoline in neutral or alkaline medium by heating with aqueous dichromate at 200° to 225° under a pressure of 320 to 350 lb. per sq. in. In another preparation of the amide, nicotinic acid is heated with molten urea at 180° to 220° in the presence of a catalyst, ammonium molybdate (210).

Sumner *et al.* (211) prepared coenzyme I from bakers' yeast by extraction with ether, alcohol, and sulfuric acid and precipitation with alcohol in the cold. Further purification by adsorption on acid-washed Norit and elution with aqueous amyl alcohol yielded a preparation two thousand times as active as the original yeast. Its elementary analysis, however, did not agree with the generally accepted formula for coenzyme I. Further criteria for the purification of this substance are described by Schlenk & Schlenk (212) who also give detailed directions for the apozymase test, catalytic hydrogenation, and the determination of monohydro-cozymase.

Huff (213) describes the synthesis and properties of a fluorescent condensation product of *N'*-methylnicotinamide and acetone, which he believes to be one, but apparently not the only, reaction product obtained in the analytical estimation of this metabolite in urine (214). The curve relating fluorescence to pH differs in the crystalline product from that obtained in the analytical method. A new metabolite, viz., the 6-pyridone of *N'*-methylnicotinamide, has been isolated by Knox & Grossman (215) from human urine to the extent of 10 per cent of the administered dose of nicotin-

amide. This compound fluoresces only slightly under the conditions employed in Huff & Perlzweig's method for *N'*-methylnicotinamide and is eliminated by the adsorption step.

A derivative of nicotinamide not hitherto reported is 2,5-dinitotinylornithine, which Dann & Huff (216) have isolated from dried chick droppings, after observing that these excreta contained more apparent nicotinic acid after alkaline hydrolysis than before. In this metabolite one molecule of nicotinic acid is attached through peptide linkage to each of the amino groups of ornithine.

Huff (217) describes a procedure for the *in vitro* conversion of trigonelline to nicotinic acid. However, after oral administration of trigonelline to human subjects, Foà & Glicksman (218) could not observe any significant increase in nicotinic acid in the urine.

Several contributions have appeared on the relation of tryptophane to nicotinic acid metabolism. Singal *et al.* (219) showed that rats receiving tryptophane excreted relatively large amounts of nicotinic acid, a methylated derivative, and especially an acid- (but not alkali-) labile precursor. Increased growth and liver-nicotinic acid levels were observed in rats on supplementing a 9 per cent casein diet with nicotinic acid or tryptophane; but the addition of a mixture of histidine, valine, and threonine, with or without lysine, depressed growth without reducing the liver nicotinic acid (220).

A rise in urinary excretion of nicotinic acid metabolites following the administration of DL-tryptophane has likewise been observed in human subjects (221, 222), in the rat (223), and in the horse and cotton rat (224). Briggs *et al.* (225, 226) reported that the growth of chickens could be depressed by feeding a nicotinic acid-low ration containing excessive amounts of protein low in tryptophane, e.g., zein or gelatin. The latter was three times as effective in this respect as the former, an observation which the authors attributed partly to the combination of amino acids present, in particular arginine, glycine, and alanine. The depressing action could be overcome by the addition of nicotinic acid or tryptophane. Krehl *et al.* (227) advanced the hypothesis that the effect of corn in nicotinic acid-low rations is due to an amino acid imbalance in its predominant protein, zein, which increases the requirement for tryptophane. They suggested that the kind of carbohydrate in the ration was a determining factor since better growth was observed with dextrin than with sucrose. It was postu-

lated that dextrin either stimulated the synthesis of additional essential nutrients or provided better conditions for bacterial synthesis in, and absorption from, the intestine. It is not necessary however to assume a specific deleterious role for corn since replacement of the casein in synthetic diets with fibrin, egg albumin, or soy globulin, by virtue of their tryptophane content, prevented the "growth-retarding" effect of corn (228). Salmon (229) has shown that the nicotinic acid requirement of the rat on noncorn rations is influenced by the level of casein, tryptophane, or fat in the diet. On a low-fat diet containing less than 18 per cent casein as the sole protein, Salmon found methionine to be the first and nicotinic acid the second limiting factor for growth in rats. Addition of tryptophane, casein, or fat tended to counteract the nicotinic acid deficiency. The tryptophane-nicotinic acid sparing action of fat is believed to be due to a shift of energy metabolism to a fat rather than carbohydrate basis, indicating that nicotinic acid, like other B vitamins, functions principally in carbohydrate metabolism.

While little light has been shed on the mechanism of the conversion of tryptophane to nicotinic acid in the animal organism, Beadle *et al.* (230) have presented evidence to show that mutant strains of *Neurospora crassa* effect the conversion by way of kynurenine (o -NH₂C₆H₄COC₂H₄COOH). A strain of this mutant (No. 65001) capable of growing in the presence of either nicotinic acid or tryptophane was grown in a medium containing just sufficient of the latter to support maximum growth. The medium was then found to be inadequate for another mutant strain (No. 3416) which required nicotinic acid. But when strain No. 65001 was grown in an excess of tryptophane, the resulting medium was capable of supporting the growth of strain No. 3416, showing that nicotinic acid had been synthesized from tryptophane. Similar observations were made when kynurenine was used instead of tryptophane thus suggesting the intermediate role of kynurenine in the conversion process. In microbiological growth experiments using media containing enormously excessive amounts of specific nutrients, it is well to keep in mind the observation of Koser & Kasai (231) that

in the presence of high concentrations of one vitamin the cells are more exacting and under such conditions fail to grow, or grow slowly, unless a varied assortment of other nutritive substances is supplied.

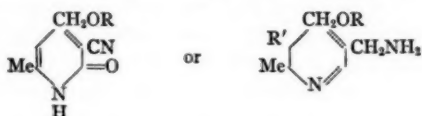
These investigators found that an increase in nicotinic acid (or nicotinamide), from the fractional microgram doses sufficient for optimal growth to the level of 1 mg. per ml. of medium, produced little effect on bacterial growth, whereas increasing the concentration in the range from 3 to 10 mg. per ml. retarded or even inhibited it completely. This effect could be prevented by an extract of yeast but to only a slight degree by a mixture of crystalline vitamins, purines, and other substances.

The results of collaborative studies of the microbiological assay for nicotinic acid by an improved technique (232), under the sponsorship of the Association of Official Agricultural Chemists, are reported by Strong (233). In the recommended method the preparation of media was simplified, the concentration of sodium acetate buffer reduced and the size of sample and time of autoclaving increased. This procedure is now described in substantially similar form in the *A.O.A.C. Book of Methods, Sixth Edition*, and in the *U. S. Pharmacopoeia XIII*. Kemmerer & Shapiro (234) compared trypsin-hydrolyzed casein, acid-hydrolyzed casein, and charcoal-treated peptone as sources of amino acids for the *L. arabinosus* assay medium for nicotinic acid. They preferred the first of these because of economy and ease of preparation and a slightly increased growth response, indicating the presence of unidentified factors not present in the other sources. A microbiological assay for nicotinic acid based on turbidimetry of yeast cultures (*Torula cremoris*) is claimed to possess the advantage of speed (sixteen to eighteen hours) over the *L. arabinosus* assay and, when combined with acid and alkaline hydrolyses, to permit differential assay of mixtures of nicotinic acid, trigonelline and *N'*-methylnicotinamide (235). Another microbiological procedure based on turbidimetry of cultures of *Proteus* HX19 is said to have sufficient sensitivity to permit measurements in the range 0.005 to 0.1 μ g. per 10 ml. of medium (236).

VITAMIN B₆ (PYRIDOXINE AND ITS DERIVATIVES)

Synthesis.—Several methods for the synthesis of pyridoxine have been described in recent patent literature. These procedures involve too many steps to be described in the limited space available. It is of interest however to note the different intermediates for these syntheses. One process (237) begins with 2-Me-3-methoxycinchonic acid; another (238) with 2-Me-4-phenoxyethyl-

5-cyano-6-hydroxypyridine-3-carboxylic acid; a third group of procedures (239) starts with compounds of the type



where R' is an alkyl or aryl group and R¹ is amino or acylamino; still another (240) involves the use of 6-Me-3,4-dicarboxy-2(1)-pyridone. Oil-soluble acyl derivatives of pyridoxine have been produced (241) by acetylation of natural extracts of the vitamin.

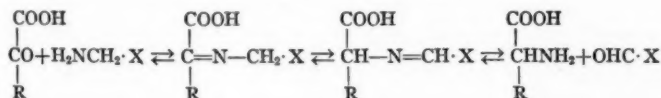
Karrer & Viscontini (242) have synthesized a codecarboxylase by attacking the phenolic group of pyridoxal acetal with phosphorus oxychloride in pyridine, and hydrolyzing the barium salt of the resultant compound with hydrochloric acid in absolute alcohol. The crystalline phosphoric ester of pyridoxal acetate was shown to possess strong capacity for decarboxylating tyrosine when dried *Streptococcus faecalis* R was employed as the apoenzyme.

Physiological activity.—Evidence has been presented against the postulated (243) existence of an unidentified acid-labile factor possessing vitamin B₆ activity for the rat and for *Saccharomyces carlsbergensis*. Involved in this problem are the relative rates of acid hydrolysis of the various forms of vitamin B₆, phosphorylated and bound to protein, and differences in their relative activities for various microorganisms, especially *S. carlsbergensis* and *S. faecalis*. Rubin *et al.* (244) found that values for the vitamin B₆ activity of yeast (for *S. carlsbergensis*) reached a peak when the pH of the one-hour extraction was kept within the range 1.5 to 2.0. Lower values obtained at higher or lower pH levels could be increased by extending the time of acid hydrolysis or by enzymatic treatment. Rabinowitz & Snell (245) point out that failure to hydrolyse pyridoxamine phosphate would yield higher results with *S. faecalis* as the test organism than with *S. carlsbergensis* for which it is inactive. (It will be recalled that of the unphosphorylated forms of vitamin B₆, *S. carlsbergensis* responds to pyridoxine, pyridoxal, and pyridoxamine, whereas *S. faecalis* responds only to the latter two.) These authors refer to the complete conversion of pyridoxal phosphate to pyridoxamine phosphate by heating with glutamic acid and offer evidence based on hydrolysis rates that

pyridoxamine phosphate is the major form of the vitamin present in dried yeast and liver powder. The relative ease of producing combined forms of pyridoxal with various amino acids (246), as shown by paper partition chromatography, suggests that further complexities may be expected in the resolution of the vitamin B₆ activity of hydrolysates of proteinaceous materials.

The role of vitamin B₆ in transaminase reactions is sufficiently well established to justify regarding this vitamin as occupying a position in protein metabolism comparable to that of thiamine in carbohydrate metabolism. Ames *et al.* (247) have analyzed various tissues of normal and vitamin B₆-deficient rats for transaminase and succinic oxidase and the influence of pyridoxal, pyridoxamine, and their phosphates on enzyme activity. They found reduced levels of transaminase in the heart and kidney of deficient rats but the succinic oxidase activity was only slightly depressed. Addition of pyridoxal or pyridoxamine, with or without adenosinetriphosphate, was ineffective but their respective phosphates, at about the same low levels, caused marked reactivation of transaminase (but not succinic oxidase) activity. Schlenk & Fisher (248) isolated glutamic-aspartic acid transaminase from pig heart and found pyridoxal phosphate, in most preparations, to be the prosthetic group, although pyridoxamine phosphate was found in a few instances. Their observations were based on a differential diazo colorimetric method for the pyridoxine, pyridoxal, and pyridoxamine (249).

Schlenk & Fisher illustrate the role of pyridoxal and pyridoxamine as prosthetic groups in the transamination process by the following equations in which X represents the ring structure of pyridoxine attached to the protein apoenzyme.



In furtherance of earlier work of Gunsalus (250, 251, 252), Gale (253), and their respective co-workers, showing that members of the vitamin B₆ group can function as coenzymes in the decarboxylation of amino acids, Braunshtein & Kritzman (254) and Gale & Tomlinson (255) have investigated the relation between transaminase (which they call co-aminopherase), codecarboxylase, and pyridoxal. They conclude that the coenzyme system of mammalian

aspartic aminopherase is either different from, or more complex than, pyridoxal phosphate and that this coenzyme system is present to some extent in the natural codecarboxylase preparation. Braunshtein has recently reviewed the subject of transamination (255a). Of interest in the relation of pyridoxine to transaminase activity is the observation of Pagé & Gingras (256) that massive doses (100 to 400 mg. daily) of glycine were toxic to pyridoxine-deficient, but not to pyridoxine-fed, rats. The possibility exists that the vitamin B₆ requirement is influenced by quantitative as well as qualitative differences in the amino acid composition of the diet.

Additional evidence for the coenzyme activity of pyridoxal phosphate may be found in the work of Umbreit *et al.* (257) on the enzyme preparation from the mycelium of *Neurospora sitophila* which converts indole plus serine to tryptophane. Loss of activity on storage of the mold tissue in the frozen state could be restored by adding pyridoxal phosphate but only in the presence of indole or serine.

Schweigert & Pearson (258) studied the capacity of vitamin B₆-deficient rats and mice to utilize tryptophane as a precursor of nicotinic acid. The pronounced effect of pyridoxine on the ability of the rat to convert tryptophane to nicotinic acid and *N'*-methyl-nicotinamide may be seen in Table I.

TABLE I
EFFECT OF PYRIDOXINE ON TRYPTOPHANE CONVERSION

Diet	μg. Excreted per Day	
	<i>N'</i> -Methyl-nicotinamide	Nicotinic Acid
Basal (B ₆ deficient)	45- 140	10- 24
Basal+Pyridoxine (2.5mμ/gm. diet)	95- 185	23- 50
Basal+Tryptophane (100 mg./day)	180- 435	16- 35
Basal+Pyridoxine+Tryptophane	810-2190	95-430

In contrast to these findings is the failure of Rosen *et al.* (259) to obtain more than a slight reversal of the progressive decrease in *N'*-methylnicotinamide excretion, which occurs in B₆-deficient rats receiving 100 mg. doses of tryptophane, after feeding supplemental pyridoxine. These authors investigated the concept that

the urinary end product of tryptophane metabolism in vitamin B₆-deficient rats and dogs is xanthurenic acid instead of kynurenic acid as in normal animals (260). Simultaneously with the disappearance of xanthurenic acid from the urine after pyridoxine dosage they observed only a slight effect of tryptophane on *N*'-methylnicotinamide excretion. Hence they believe that the two processes are not directly related. Kynurenine was suggested by Lepkovsky *et al.* (260) as the intermediate in the metabolic transformation of tryptophane in both normal and B₆-deficient animals. But when Rosen *et al.* (259) administered kynurenine, kynurenic acid, or xanthurenic acid to rats on a complete diet they found no rise in *N*'-methylnicotinamide excretion, suggesting that the kynurenine pathway is not involved in the conversion of tryptophane to nicotinic acid in the rat.

PANTOTHENIC ACID

The new coenzyme A of Lipmann, involved in the acetylation of aromatic amines in liver preparations and in the acetylation system for choline in brain, has been found to be a pantothenic acid derivative (261). Yet another important role of this coenzyme appears to be in a reaction which occurs in the liver between acetate and adenylypyrophosphate with the formation of a compound resembling acetylphosphate (262). Novelli & Lipmann (263) have reported the bacterial synthesis of coenzyme A from pantothenic acid with *Lactobacillus arabinosus* and *Proteus morganii*. Respiratory stimulation of deficient *Proteus morganii*, following the addition of pantothenic acid, was accompanied by a parallel increase in coenzyme A concentration. This is interpreted as confirming earlier observations that pantothenic acid permits pyruvate oxidation to proceed beyond the acetate stage to completion (264, 265).

The observation that high casein diets tend to diminish the requirement of the rat for pantothenic acid has been further investigated and confirmed in urinary and fecal excretion studies by Nelson *et al.* (266). Neither increased intestinal synthesis nor decreased renal loss of pantothenic acid could account for this sparing action of high casein diets. Whether the protein furnishes a precursor (cf. the tryptophane-nicotinic acid relationship) or whether some factor involving pantothenic acid utilization is concerned in this phenomenon remains to be investigated. This recalls the suggestion of Abelin (267) that β -alanine and D-valine may be physio-

logical precursors of pantothenic acid since these amino acids, like the vitamin itself, reduce the effect of thyroxine dosage on experimental hyperthyroidism.

By blocking the enzymic decarboxylation of aspartic acid to β -alanine, cysteic acid prevents the bacterial (*E. coli*) synthesis of pantothenic acid [Ravel & Shive (268)]. Either β -alanine or pantothenic acid prevents the toxicity of cysteic acid up to levels of 30 mg. of inhibitor per 10 ml. above which its toxicity is irreversible. Aspartic, glutamic, and α -ketoglutaric acids competitively inhibit the toxicity of cysteic acid toward *E. coli* and other microorganisms. In this connection reference may be made to the work of Hartelius (269) showing that glutamic and aspartic acids, glutamine, and asparagine promote the growth of *S. cerevisiae* only in the presence of optimal concentration of β -alanine, below which they are growth-inhibitory. The antivitamin action is attributed to chemical combination of these compounds with β -alanine forming dipeptides; citric, malic, succinic, tartaric, and pyrrolidone-carboxylic acid failed to show this effect.

PTEROYLGLUTAMIC ACID

Synthesis.—A second synthesis of pteroylglutamic acid (liver *L. casei* factor) and pteric acid has been reported by the Lederle group (270). The synthesis of pteroylglutamic acid (PGA) was achieved by heating a mixture of *N*[(2-amino-4-hydroxy-6-pteridyl) methyl] pyridinium iodide, *p*-aminobenzoyl glutamic acid and sodium methylate in ethylene glycol solution at 140° for three hours. A product containing 15 per cent biologically active material was obtained. By using *p*-aminobenzoic acid in place of *p*-aminobenzoylglutamic, a compound was obtained which was active for *S. faecalis* R but not for *L. casei* and the chick. In December, 1947, at the New York Academy of Sciences, this group of investigators described the synthesis of the fermentation *L. casei* factor, pteroyl- γ -glutamyl- γ -glutamylglutamic acid.

Wittle *et al.* have reported on the oxidative degradation of PGA with sodium chlorate or permanganate (271). The same group of workers also prepared "dihydrovitamin B₆" and, because of its easy reoxidation in air, suggest that the vitamin may function as a hydrogen acceptor in some enzyme systems (272).

The *Streptococcus lactis* R (better known as *S. faecalis* R) factor of Stokes *et al.* (273) has been isolated by Rickes *et al.* (274) from

Rhizopus nigricans and designated rhizopterin. Treatment with acid or alkali gave a pterin-like degradation product, aporhizopterin, which has the same chromophoric group as vitamin B₆ (275). By degradation and synthesis the structure of rhizopterin has been found to be *p*-[*N*(2-amino-4-hydroxypyrimido(4,5-*b*)pyrazin-6-yl-methyl)-formamido] benzoic acid. One synthesis involved the formylation of pteric acid.

Rhizopterin is highly active for *S. faecalis* R but inactive for *L. casei* and the chick. Granulocytopenia induced in rats fed succinylsulfathiazole on a PGA-deficient diet could not be cured by rhizopterin.

Antagonists.—Lampen & Jones (276) showed that the primary inhibitory action of the sulfonamides is directed against the synthesis of PGA via *p*-aminobenzoic acid. Under the conditions employed organisms differed in their sensitivity to sulfonamides according to their requirement for PGA. Enterococcus strains which require the preformed PGA were relatively insensitive to sulfonamides whereas those able to synthesize it were sensitive only in the absence of the preformed nutrilitate.

According to Martin and co-workers (277) the activity of PGA for *S. faecalis* R and *S. aureus* is counteracted by its D(-)-7-methyl derivative ("methylfolic acid") which not only displaces the vitamin but interferes with its synthesis at the stages of pteric acid formation and union with glutamic acid.

A crude preparation containing the L-analogue (since *p*-aminobenzoyl-L-glutamic acid was used in the synthesis instead of the D-compound) was found by Franklin *et al.* (278) to antagonize the effect of PGA not only for these microorganisms but for rats. In fact the syndrome produced with this preparation supplementing a PGA-deficient diet was even more acute than that resulting from the diet supplemented with succinyl-sulfathiazole. The effect was reversible however. These investigators were also able to induce symptoms in chicks and in mice by including the crude preparation of the antagonist in PGA-deficient diets (279).

Pteroylaspartic acid has been synthesized by Hutchings *et al.* (280) and found to be antagonistic to pteroylglutamic acid and certain of its derivatives.

Action.—Hall (281) has compared the effects of synthetic PGA and of R. J. Williams' "7 per cent folic acid" concentrate from spinach in respect to acid production by *S. faecalis* R over short

and long incubation periods. On the basis of relative differences in activity during the early and late phases of growth he suggests that the natural folic acid is of a dual nature. In this connection more consideration must be given the possibility of a time lag for the hydrolysis of conjugates in natural concentrates. One is also inclined to question results based on prolonged incubation which may entail changes in the media caused by the accumulation of bacterial metabolites (despite periodic pH adjustment) or by the development of adaptive mechanisms during growth. It will be recalled that organisms responding to initial growth stimulation by streptogenin are ultimately able to grow as well without an exogenous source of this factor.

By growing *L. arabinosus* in the presence of excess *p*-aminobenzoic acid, Sarett (282) found that synthesis of pteroylglutamic acid occurred. However PGA and pteric acids only partially replaced *p*-aminobenzoic acid for growth of these organisms which, it is suggested, may require *p*-aminobenzoic acid for purposes other than synthesis of PGA.

The relation between PGA and thymine still remains to be clarified. Stokes (283) showed that "folic acid," while not itself replaced by thymine, was utilized by certain lactic acid organisms in the production of a thymine-like compound; and that the acid-producing activity of thymine in *S. faecalis* R cultures was not as great as that of folic acid. However, by prolonging the incubation period Hall (284) demonstrated that ultimately as much acid is produced in one case as in the other. He also observed a synergistic relation between folic acid and both thymine and orotic acid. From these observations Hall suggests that thymine may actually be the precursor of folic acid, or that thymine may participate in some alternate metabolic course. That intermediary thymine synthesis is not a *sine qua non* of PGA activity has been known from the work of others.

Evidence showing that thymine cannot replace PGA in the rat has been reported by Petering & Delor (285). When fed at dosages equivalent microbiologically to as much as 7.2 μ g. of PGA, thymine was ineffective in curing or preventing the growth or hematologic syndrome. These authors conclude that thymine is not a true substitute for PGA, and any activity of this sort shown by thymine, pyrimidines, etc., must result from some indirect mode of action.

Supplementing earlier work, which indicated a beneficial effect

of the *L. casei* factor on lactation in rats, Cerecedo & Miron (286) have made similar observations in mice. Nelson & Evans (287) have reported marked improvement in maternal body weight and in the maternal leucocyte picture when high levels of synthetic PGA (110 $\mu\text{g. per 100 gm. diet}$) were added to purified rations. Higher levels (275 $\mu\text{g.}$) were necessary for improvement in weaning weight of the pups. A liver eluate powder, fed in equivalent PGA concentrations, induced significantly greater improvement, suggesting that an additional unknown factor may be necessary during this period.

In paired-feeding studies with eight-week old pigs, Cunha *et al.* (288) fed folic acid (or *p*-aminobenzoic acid) alone or in combination with inositol and biotin as supplements to a purified basal ration containing the other known B-complex vitamins. No beneficial effect was observed on appearance, growth, or feed utilization but some stimulation of hemoglobin formation was noted. A favorable effect on growth resulted from folic acid supplementation of a ration of natural grains, but in this case no improvement occurred in hemoglobin formation.

Assay.—The variations in response of microbial species to different conjugated forms of PGA make it desirable to supplement microbiological assay data with bioassays on animal species. Little & Briggs (289), using the chick as the assay animal, have estimated the folic acid activity of the common grains, protein concentrates, and other substances. The richest source, alfalfa meal, was found to contain 11.3 $\mu\text{g. per gm.}$, while grains and animal protein concentrates had very little activity. Liver and yeast contained approximately 50 $\mu\text{g. per gm.}$

Villela (290) has proposed a method for the estimation of PGA based on its fluorescence in neutral and alkaline solution but the specificity seems questionable and its applicability to extracts of natural foods was not demonstrated. The Lederle group (291) described a chemical method for estimating PGA and related compounds based on cleavage of the molecule by reduction in an acid medium to yield a pteridine and *p*-aminobenzoylglutamic acid. Aromatic amine is measured colorimetrically before and after reduction by the diazotization method of Bratton & Marshall (292), the difference being a measure of the pteroyl derivative present. The method may be useful in assaying pharmaceutical preparations but is limited in its applicability to concentrates de-

rived from natural sources since the active compound must be present in at least 5 per cent concentration. Furthermore, any compound which, after reduction, would give the diazo color reaction with *N*-(1-naphthyl)-ethylenediamine would interfere and the distribution of such compounds in nature is not known.

The studies by Kidder and his associates (293, 294) on the biochemistry of the ciliated protozoan, *Tetrahymena geleii* W, have revealed that this organism not only requires PGA for growth but responds more actively to its conjugated forms. The concentrations, in μg . PGA per ml. of medium, for half-maximum response were as follows: PGA 0.00064; pteroyltriglutamic acid 0.0005; pteroylheptaglutamic acid 0.00032. Xanthopterin, *p*-aminobenzoylglutamic acid, pteric acid, and methyl pteridine were inactive either alone or in combination. Comparative assays of natural products with chicks (or other animals) and *Tetrahymena* are awaited with interest since current microbiological methods involving preliminary enzymatic cleavage of conjugated forms of PGA cannot be expected a priori to furnish a safe measure of available PGA in animals. The situation is further confused by conflicting evidence regarding the ability of the chick to utilize the two best recognized conjugates of PGA. Thus Jukes & Stokstad (295) observed that chicks utilized PGA and pteroyltriglutamic acid equally well, on a molar basis, for growth and hematopoiesis, in contrast with the findings of Scott *et al.* (296) who later (297) suggested that the lactone of 4-pyridoxic acid functioned in an enzyme system to effect breakdown of folic acid conjugates. However, when they attempted to repeat the experiments of Scott *et al.*, Jukes & Stokstad could observe no improvement in response upon supplementing pteroyltriglutamic acid with the 4-pyridoxic acid lactone.

Moore *et al.* (298) found high test doses of folic acid to be poorly stored in the liver of the chick and only slightly, if at all, in muscular tissue. Levels of folic acid in the chick were found to be highest in the liver, kidney, and pancreas and lowest in the skin and muscles. Franklin *et al.* (299) demonstrated that the major part of the PGA in the tissues of chicks fed purified diets was in conjugated form; liver tissue at three to four weeks contained 0.7 to 1.9 μg . of free PGA per gram but after hydrolysis with chicken pancreas as a source of "conjugase," the range of values was 11.3 to 14.0 μg .

Taylor (300) has studied the folic acid requirement of hens by supplementing a basal ration of polished rice, sardine meal, casein, vitamins, and minerals with synthetic PGA. He found the low level of 12 $\mu\text{g.}$ per 100 gm. to suffice for egg production; higher levels were needed for sustained optimum hatchability but minimum limits could not be stated. In the previous year Robertson *et al.* (301) reported requirement levels ranging from 25 $\mu\text{g.}$ of folic acid per 100 gm. ration for survival to six weeks, to 55 $\mu\text{g.}$ for optimum feathering. It would appear that more uniform assay procedures may reconcile the divergence between these two series of observations.

It has been demonstrated that PGA is twice as effective when administered intramuscularly as when given by mouth (302, 303).

The twenty-four hour urinary excretion of free PGA in normal human subjects was found by Steinkamp *et al.* (304) to average 2.34 $\mu\text{g.}$ by *S. faecalis* assay. After oral dosage of 5 to 16 mg. the recovery averaged 28.5 per cent in normal subjects but considerably less in chronic hospitalized patients.

Schweigert & Pearson (305) have studied the free and conjugated folic acid content of the whole blood and plasma of various species. The ranges and mean values (in parentheses) for the free acid in $\text{m}\mu\text{g.}$ per ml. whole blood were: human 0.5 to 1.3 (0.85); chicken 4.4 to 16.5 (8.7); turkey 10.2 to 27.6 (16.8); horse 2.2 to 6.0 (3.3); cattle 0.6 to 4.5 (1.9); pig 4.5 to 9.9 (6.6). Lower levels were found in plasma but these, too, varied with the species. After takadiastase digestion the whole blood levels for the chicken, pig, and especially for humans and cattle, increased markedly to a range of 20 to 40 $\text{m}\mu\text{g.}$ per ml.

In a preliminary report on the "folic acid" content of certain foods, Olson *et al.* (306) classify them as follows: Very high: fresh deep green leafy vegetables, liver; High: fresh green vegetables, cauliflower, kidney; Medium: beef, veal, dry breakfast cereals prepared from wheat; Low: root vegetables, tomatoes, cucumbers, light green leafy vegetables, bananas, pork, ham, lamb, cheese, milk, dry breakfast cereals prepared from rice or corn, and many canned foods. Noteworthy is the correlation of folic acid with the chlorophyll content of plants. These authors found the folic acid content of vegetables to diminish rapidly on storage at room temperature but not at refrigeration temperatures. The majority of foods that compose the diets of Cuban patients with tropical

sprue have been found by Angulo & Spies (307) to be remarkably low in folic acid. These include cassava, corn meal, plantain, rice, and sweet potato. Schaefer *et al.* (308) have investigated the folic acid and other vitamin requirements of foxes on purified rations.

BIOTIN

The ubiquitous distribution of biotin and its high degree of biological activity for microorganisms and tissue cells, and the existence of natural antagonists, continue to constitute a challenge to biochemists who visualize a profoundly important role for this vitamin and far-reaching possibilities for natural and synthetic antivitamins.

Space does not permit more than mere citation of the many recent papers on the synthesis of biotin and its intermediates, and of related compounds possessing biotin or antibiotin activity (309 to 315). Special reference must be made to the series of papers in Vol. 12 of the *Journal of Organic Chemistry* from the Lederle Laboratories at Pearl River, N. Y.

Brown & du Vigneaud (312) prepared the thiourea analogue of desthiobiotin to determine the effect on biological activity of alterations in the imidazolidone moiety. This analogue, in contrast to desthiobiotin itself, showed little growth activity for *S. cerevisiae* and low antibiotin activity for *L. casei*.

Dittmer & du Vigneaud (316) investigated the relative anti-biotin activities of 2-oxo-4-imidazolidine-valeric, -caproic, -heptanoic, and -octanoic acids against yeast and *L. casei*. Maximal activity was found in the caproic acid derivative, which differs from desthiobiotin in lacking the CH_3 group. Biotin counteracted this activity in every case but yeast growth was more strongly inhibited by these oxoimidazolidine aliphatic acids when desthiobiotin, a possible precursor of biotin, was the growth stimulant instead of biotin itself. Rogers & Shive (317) found that 2-oxo-4-imidazolidine caproic acid inhibited the growth of *E. coli* by competing with desthiobiotin for an enzyme system essential for the biosynthesis of biotin. Hofmann *et al.* (318) prepared the sulfonic acid analogues of oxybiotin and homooxybiotin and found the former to have antibiotin and antioxybiotin activity while *dl*-homooxybiotinsulfonate showed some stimulatory action. By treating with Raney nickel, biotin is converted to desthiobiotin,

while under the same conditions oxybiotin is unaffected. Desthiobiotin is inactive for *L. arabinosus* while oxybiotin is active. Thus assays before and after Raney nickel treatment can serve as a basis for quantitative differentiation of biotin and oxybiotin in mixtures. Using this method, as well as other differential assays, Axelrod *et al.* (319) were unable to find biotin in yeast cells grown in oxybiotin media, in contrast to the report of Rubin *et al.* (320) that oxybiotin was quantitatively converted to biotin during the growth of *S. cerevisiae*.

Since avidin-combining capacity frequently parallels antibiotic activity, special interest attaches to the method proposed by Wright & Skeggs (321) for measuring affinity for avidin. It is based on varying the amount of analogue added to a stoichiometric mixture of biotin and avidin. The relative affinity for avidin is expressed as that ratio of concentration of analogue to biotin at which one-half of the latter remains free and available for growth of the test organism. The method is more sensitive and requires less material than the procedure based on the capacity of analogues to displace biotin from combination with avidin. Of nineteen compounds having some structural similarity to biotin, Wright *et al.* (322) found only three with affinity for avidin: γ -(3,4-ureylenecyclohexyl) butyric acid, δ -(2,3-ureylenecyclohexyl)- and δ -(3,4-ureylenecyclohexyl) valeric acids. None of the compounds studied showed biotin or antibiotic activity for *L. arabinosus*.

In view of the earlier work of Williams & Fieger (323) demonstrating the stimulant effect of oleic acid on the growth of *L. casei* in a biotin-free medium, Trager's finding (324) of a fat- and ether-soluble fraction in plasma which can replace biotin for the growth of lactic organisms is particularly interesting. This substance is not a fatty acid nor is it inactivated by avidin, yet when injected in chicks it is protective against egg-white injury. A vaccenic acid fraction (m. p. 38°, iodine no. 84.5), as well as oleic and elaidic acids, was investigated by Axelrod *et al.* (325) for biotin-like activity. The activity of these acids for *L. casei* was equivalent, respectively, to 5.3, 11.5, and 15.2 μ g. of biotin per gm., but they were less potent for *L. arabinosus* and inactive for *S. hemolyticus* and *S. cerevisiae*.

Observations on the oleic acid requirement of various lactic acid bacteria have been extended by Williams *et al.* (326). Most lactic acid organisms employed in microbiological assay work

manifest a requirement for oleic acid only when the media lacks biotin. In the presence of the surface-active solubilizing agent Tween 40 (the palmitic acid ester of polyethylene derivatives of sorbitan) the effectiveness of oleic acid is greatly enhanced and its toxicity prevented. Tween 80, the oleic acid counterpart, behaves like Tween 40 plus oleic acid. Since biotin appears to function in the synthesis of both oleic acid and aspartate, lactic acid bacteria do not require biotin when adequate amounts of these substances are supplied preformed.

A stimulatory action of oxaloacetate on the growth of *L. arabinosus*, in media deficient in biotin and aspartic acid, has been shown by Lardy *et al.* (327). They suggest that oxaloacetate is transaminated to aspartate and explain the failure of biotin-deficient organisms to synthesize aspartate (328) by their inability to condense pyruvate and carbon dioxide to form oxaloacetate.

The difficulty of conducting vitamin balance studies in animals in which intestinal synthesis by bacteria is a significant factor is emphasized by work conducted during 1940-42 but only recently reported by McGregor *et al.* (329). The excess biotin eliminated in the urine (5 to 40 per cent of the total) and feces (60 to 95 per cent) over that ingested in rations containing raw egg white ranged from 0.6 to 9.4 μ g. per day. Bacterial synthesis occurred chiefly in the cecum and large colon.

The carcinogenic action of *p*-dimethylaminoazobenzene in rats was accelerated by biotin supplementation of a diet protective against tumor development, but when the diet was favorable to tumor growth, such acceleration was not observed (330).

CHOLINE

Borglin's (331) studies of choline deficiency in rats revealed that while 1.5 mg. per day sufficed for normal growth and protection against the kidney lesion, larger amounts (approximately 5 mg.) were required for lipotropic action. Regardless of the diet used, less than 0.5 per cent of the ingested choline was found in the urine. Similar low excretion ratios were found in human adults and infants. The difficulties attending the interpretation of data such as these are emphasized by the various interrelations between choline and other nutrients. For example, physiological implications of the role of choline as a methyl donor are suggested by the work of Salmon (229) on the interrelationships of protein, fat,

choline, methionine, cystine, nicotinic acid, and tryptophane. Worthy of mention here is the observation that dietary choline and nicotinic acid increased the efficiency of protein utilization by relieving the demand on methionine for labile methyl groups and on tryptophane as a precursor of nicotinic acid (*q.v.*).

Histopathological studies have been made to determine the nature of the renal lesions observed in rats on choline-deficient diets. That the principal involvement is in the tubules is indicated by the reports of Dessau & Oleson (332) and of Hartcroft & Best (333). The former suggest that the vascular disorder results from interference with venous outflow rather than from a disturbance of the arterial supply. Within a few days after young rats were placed on a choline-deficient diet, the appearance of fatty droplets in the cells of the proximal convoluted tubules was noted (333), and these increased in size and number reaching a maximum on the sixth or seventh day when congestion, hemorrhage, and cortical necrosis were apparent. The etiology of this phenomenon and especially its relation to loss of lipase activity in the tubules (334) await further study.

The reversible hemolytic anemia which he observed in dogs receiving choline as a supplement to a high-fat diet is believed by Davis (335) to be due not to the lipotropic action but rather to the pharmacologic or vasodilator effect of choline. This investigator has postulated that choline tends to depress erythropoiesis in bone marrow while the high fat diet stimulates hemolytic destruction of the red cells. However Clarkson & Best (336) were unable to reproduce this anemia in dogs receiving large doses of choline either with or without fat.

Of further interest in relation to the functional role of choline are the observations of Abdon & Borglin (337) with an ox-spleen extractive which corrected the impaired metabolism of minced muscles from choline-deficient rats but was without effect on muscles from the choline-fed controls. The active principle has certain physical and chemical properties resembling the acetylcholine precursor and is believed to be a choline complex.

Severe choline-deficiency in rats has been shown by Neuman & Riley (338) not to impair the uptake of isotopic phosphorus into their bones, which grew normally despite the general inanition.

In studies of the lipotropic effect of choline and inositol on cholesterol-fed rats, Ridout *et al.* (339) observed a gradually di-

minishing effect of inositol whereas that of choline was greater and persisted throughout the experimental period of sixteen weeks. A synergistic lipotropic effect of choline plus inositol was observed in these animals but no preferential effect of inositol in diminishing cholesterol esters was seen. In thiamine-deficient, cholesterol-fed rats the lipotropic effect of choline was likewise consistently greater than that of inositol, regardless of the fat content of the diet.

Du Vigneaud *et al.* (340) fed labeled sarcosine (as N¹⁵ deuteriosarcosine) and observed slow transmethylation to choline and creatine. Ringrose & Davis (341) have reported confirmation of the observations of Lucas *et al.* (342) that laying hens are capable of synthesizing substantial amounts of choline under conditions of low choline and methionine intake. Bethke *et al.* (343) likewise were unable to improve hatchability by the addition of choline or methionine, singly or together, to an all-plant-protein basal ration. Significantly better hatchability resulted from the addition of various sources of animal protein or of liver extract.

The choline content of cow's colostrum was found by Waugh *et al.* (344) to be high (0.69 mg. per ml.) and to diminish to about one-fifth in subsequent milkings; calf blood levels of choline were low at birth but increased during the first week.

NEW AND MISCELLANEOUS FACTORS

Strepogenin and factor S.—In 1940 a new growth essential for chicks was described by Schumacher *et al.* (345) which was subsequently found to resemble strepogenin in its distribution and properties. Like strepogenin it functions in maintaining an optimum rate of growth at the early stage of incubation. *L. casei* has been reported by Scott *et al.* (346) to require (in addition to strepogenin) glutathione and a factor contained in certain animal products, including whey, liver, and fish press-water. It differs from strepogenin in that it can be adsorbed on charcoal at pH 3.0 and eluted by alcoholic ammonia, and in its stability to heat and oxidation. The authors suggested that this may be the "animal protein factor" required for normal reproduction in hens. The Cornell group (347) have recently extended their studies in this direction using purified diets containing protein of low-strepogenin value. The essentiality of factor S is reaffirmed. On deficient diets chicks failed to grow, became anemic, and generally weakened and

died before they were eight weeks old. The factor-S activity of yeast, fish-meal, and crude casein appears to be correlated with their strepogenin content (determined microbiologically) so closely as to suggest the probable identity of these factors.

Woolley (348) has suggested that the strepogenin grouping in insulin is located at the end of the peptide chain and that glycine is the end-amino acid of strepogenin. This view is based on the loss of activity, on mild hydrolysis, of the derivatives of insulin resulting from reacting its free amino acids with dinitrofluorobenzene or with nitrous acid. If strepogenin were not at the end of the chain, activity of the hydrolysate would not have been affected. Similar results were obtained in the case of trypsinogen, but casein, another rich source of strepogenin, lost only about half its activity under these conditions.

Cow manure factor.—Continuing their investigations of the cow manure factor, Rubin and associates (349) have found it in the feces of hens but not in those of three to six-week-old chicks. Their suggestion that synthesis may occur in the feces after voiding was investigated by McGinnis *et al.* (350) who found practically no activity in freshly voided and immediately frozen feces but appreciable synthesis after seventy-two hours' incubation at 30°. The active factor of cow manure is transmitted by the hen to the egg where it is concentrated principally in the acetone-insoluble fraction of the yolk (351). Cow manure is equally active whether dried at 45° for twenty-four hours or sun-dried for two to three days (352).

The presence in cow manure of orally available androgenic hormone has been studied by Turner (353) in the light of its possible relationship to the growth of chicks and to the onset of egg production. The effects observed in both cockerels and pullets suggested sexual precocity rather than retardation. In any case, the androgenic hormone (whose activity could be destroyed by drying the manure at 80° for twenty-four hours) was responsible in part for the observed growth stimulation. These findings are in contrast to those of Whitson *et al.* (354) who found egg production impaired over a forty-eight-week period when cow manure was included in a good, practical ration. No observations were reported, however, on the time of onset of egg production.

The inhibitory effect of a 70 per cent soybean-oil meal diet on the growth of chicks was counteracted by feeding cow manure

(355). The active factor of cow manure is thus believed to have little or no effect on the heat-labile trypsin inhibitor in raw soymeal, which was crystallized by Kunitz (356), but rather on a property of the soymeal not influenced by heat. Supplementation with methionine instead of cow manure proved to be only partially effective in counteracting the soymeal inhibition. This is insufficient ground, however, for denying the validity of the view (357) that the efficiency of utilization of soymeal is dependent upon the rate of release of individual essential amino acids during digestive breakdown and their mutual supplementary relationships. Supplying a limiting amino acid in the free form may result in its rapid absorption but not in its optimum availability for supplementation of the amino acids released subsequently in the digestive process.

Further work on the relation of the cow manure factor to gonadotropic hormones and to protein nutrition is awaited with keen interest.

Guinea pig antistiffness factor.—Following the demonstration of a lowered concentration of adenosine tri- and diphosphates in the tissues of guinea pigs deficient in the antistiffness factor, (358, 359), van Wagendonk & Simonsen (360) showed derangements in the respiratory rate and in anaerobic glycolysis in liver, kidney, and muscle tissue of deficient animals. These alterations could be reversed by administration of 2 μ g. of the crystalline factor or by *in vitro* addition of adenosinetriphosphate to the metabolizing tissue. Coincident with the drop in the easily hydrolyzable phosphate fraction in liver and kidney, a decrease in nucleotides and nucleosides has been noted (361) while the ratio of nucleotide to nucleoside was increased. These changes were attributed to dephosphorylation of the adenylic acid system and were reversible by continuous administration of the antistiffness factor. Special interest attaches to these observations in view of the paucity of data on the distribution of the products of purine metabolism in vitamin deficiency conditions.

The deposition of calcium phosphate in the tissues of guinea pigs showing the stiffness syndrome is believed not to be a primary effect of the deficiency, but rather dependent on (a) the increased concentration of inorganic phosphate in the tissues and blood resulting from disturbance of the adenylic acid system, and (b) the lowered concentration of plasma albumin resulting from de-

ranged phosphorus metabolism in the liver (362). An increase in the blood level of nondiffusible calcium (colloidal calcium phosphate complex) was observed in deficient guinea pigs.

Some 59 sterols, their esters, and related compounds were tested for antistiffness activity by Oleson *et al.* (363). Certain esters of ergostanol were found to have the greatest activity, the acetate being about as potent as the crystalline factor isolated by van Wagtendonk & Wulzen (364) from cane sugar juice. The completely saturated ergostane carbon skeleton appeared to be necessary for maximum activity; thus ergosterol, cholestanol, cholesterol, bile acids, estrone, vitamins D₂ and D₃ were among the inactive steroids tested.

"Unidentified nutrients" in milk.—Cary & Hartman (365) have summarized their evidence for the presence of an unidentified factor (or factors) in milk, muscle, liver extract, etc., which enhances the growth of rats on ad libitum feeding of diets containing extracted casein or heat-coagulated egg albumin as the principal sources of protein. The foods lacking this factor included wheat flour, corn meal, soybean, and linseed oil meals; it was present in fluid or dried milk, cheese, casein purified by dialysis or repeated washing, egg yolk, beef and pork muscle, and in certain hays and grasses. Inasmuch as the protein sources "lacking" this factor were either deficient in one or more of the essential amino acids or were exposed to extraction or dehydration processes which may have rendered certain of them less available, the evidence presented for this "unidentified nutrient" does not appear to be entirely convincing. The possibility that reduced growth-promoting value may have resulted from denaturation of protein has not been excluded. The supplementary effect of lysine or of a hydrolysate of inactive casein on the biological value of this casein or of the heat-coagulated egg albumin used in these studies would be of interest, despite the evidence of depressed growth when the dietary levels of these proteins were increased.

However, this is not to deny the existence of nutrients in milk that have yet to be identified. The distribution of the factor described by Cary & Hartman (365) bears some resemblance to that of a stimulatory factor for *S. faecalis* R and the monkey anti-anemia factor recently reported by Ruegamer *et al.* (366). Raw milk (whole, skim, or whey), raw or lyophilized liver, and liver extract (Lederle D1432) were active both microbiologically and

in the monkey assay, whereas brewers' yeast, corn grits, and liver powder were among the inactive materials tested.

Miscellaneous.—Possibly owing to the late war, several papers from Japan on vitamins L_1 and L_2 have not previously been mentioned in this *Review* (367, 368). Vitamin L_1 was extracted from cow liver and L_2 from dried yeast in concentrated forms (176 and 250 mg., respectively, per 100 gm. starting material). Deficiency of these vitamins in rats and mice results in lactation failure through hypophyseal dysfunction. Anthranilic acid and adenylothiomethylpentose exhibited activity characteristic of vitamins L_1 and L_2 , respectively. *p*-Aminobenzoic acid was devoid of vitamin L_1 activity and adenine of vitamin L_2 . In view of the presence of such complex materials as polished rice, fish protein, and yeast extract in the diets employed by these workers and later developments in connection with intestinal synthesis by microorganisms, the question of the identity of these vitamins must await further studies with purified diets and crystalline sources of the B vitamins.

Jaffe & Elvehjem (369) have reported a growth-stimulating effect of liver extracts in rats fed a synthetic and, especially, a corn-soy diet containing "ten B vitamins and cystine" (although only nine are listed, riboflavin probably inadvertently being omitted). The active material was adsorbed on and eluted from norite; it was soluble in alcohol but not in butanol. Similar growth-promoting activity was shown by fish press-water and by commercial preparations of the liver antipernicious-anemia factor.

The absorption and metabolism of *meso*-inositol in the rat have been investigated by Wiebelhaus *et al.* (370). Less than one per cent of a 250 mg. dose was recovered in the urine collected over a twenty-four to twenty-eight-hour period. Although inositol functioned antiketogenically, no increase in liver glycogen was found after dosage. Of the various tissues examined only the heart showed any appreciable rise in inositol content after oral administration to fasting rats. Herrmann (371) found that after feeding 0.5 gm. of inositol daily for about two months, old hens showed a definite diminution in the cholesterol level of the blood and tissues.

The Cornell group have described (372) a new type of bone deformity in chicks, viz., a twisting of the tibia at the proximal end around the longitudinal axis. The incidence of this condition

was decreased by the addition of fish meal or gelatin, but glycine and/or arginine were responsible only in part for their beneficial effect.

Several recent publications on vitamin assay methodology deserve mention: *Methods of Vitamin Assay*, prepared and edited by The Association of Vitamin Chemists (Interscience Publishers, Inc., New York, 1947); *Estimation of the Vitamins*, edited by Dann, W. J., and Satterfield, G. H. (The Jaques Cattell Press, Lancaster, Pennsylvania, 1947); *Practical Physiological Chemistry*, Hawk, P. B., Oser, B. L., and Summerson, W. H. Chapter 35 on "Vitamins and Deficiency Diseases" (The Blakiston Co., Philadelphia, 1947).

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CLINICAL ASPECTS OF VITAMINS¹

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Throughout the vast literature of biology, chemistry, and physiology, there is a multitude of reports concerning various organic substances which are defined as being essential to one or more species. The isolation and synthesis of many of these factors and the determination of their role in nutrition have been accomplished with amazing rapidity. Upon the once narrow field of vitamin research have converged many separate branches of the biological and natural sciences. When it is considered that vitamins are now essential tools for investigation not only in the broad domain of human and animal nutrition but also in the diverse fields of chemotherapy, bacteriology, mycology, genetics, and enzyme chemistry, no apology is needed for want of comprehensiveness. In the immediate future, the greatest progress probably will be made in differentiating the various members of what is now termed the vitamin B complex and in determining their exact role in nutrition. Perhaps the simplest definition of the vitamin B complex is that, exclusive of ascorbic acid, it is a composite of all the water soluble vitamins found in yeast and in liver. Included among the vitamins is a heterogenous conglomeration of compounds whose structures differ greatly and whose functions, as far as known, differ even more. Their common features are that they are not built up by the body, at least in the amount needed, and that they carry on their several functions when present in small concentration or only in traces. With the new investigations which make use of techniques in many sciences heretofore sharply separated, advances in the laboratory have out-paced those in the clinic. We physicians are now at the stage where we have more vitamins than recognized diseases for which to use them. In order to dissect the symptoms due to a depletion of a single factor from the confusing mixture of symptoms characterizing the naturally occurring multiple deficiency, studies have to be made on human beings. Such studies have been, for the most part, of relatively short duration so that we have much more data on the manifestations of an isolated acute

¹ This review covers the period from January, 1946 to December, 1947.

deficiency than of the chronic partial deficiency. Nevertheless, the progress being made in a number of clinics bears promise of more clarity for future knowledge.

Advances in the field of vitamins have come so rapidly and are of such far-reaching importance that a paper of this length cannot be comprehensive. Accordingly, this report will be concerned chiefly with thiamine, nicotinic acid, riboflavin, ascorbic acid, folic acid, choline, dicoumarol, *p*-aminobenzoic acid, rutin, and vitamins A, D, K, and the tocopherols. Such important chemical and physiological substances as pyridoxine, pantothenic acid, inositol, biotin, adenylic acid, vitamin P, extrinsic factor, xanthopterin, and α - and β -pyracin will not be discussed here as there is inadequate clinical evidence to support claims as to their therapeutic usefulness in human beings.

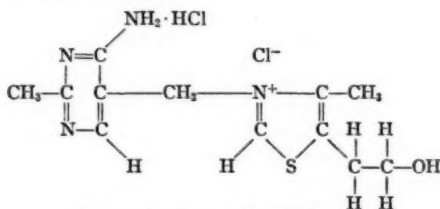


FIG. 1.—THIAMINE HYDROCHLORIDE

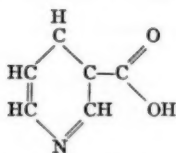


FIG. 2.—NIACIN

THIAMINE

Thiamine holds a place of honor because it was among the first of the vitamins to be isolated in pure form and among the first to be made *de novo*. Also, it is of prime importance in the prevention and relief of beriberi, which has the most ancient history of all deficiency diseases. Thiamine (Fig. 1) has a universality of function in living cells. Anatomically and clinically the lack of thiamine in human beings is followed by widespread neuritis and fatty degeneration of the myelin sheaths. A very rare manifestation of

beriberi is called beriberi heart disease. The Oriental concept of beriberi heart disease, emphasized by Wenckebach and his associates (47), has dominated medical thought. Blankenhorn *et al.* (1) have studied twelve cases in the past five years which conformed to the following criteria: (a) insufficient evidence of other etiology; (b) three months or more on a thiamine-deficient diet; (c) signs of neuritis or pellagra; (d) enlarged heart with sinus rhythm; (e) dependent edema; (f) elevated venous pressure; (g) minor electrocardiographic changes; and (h) recovery with decrease in heart size or necropsy evidence of beriberi heart disease. Clinical improvement following rest or treatment with thiamine is characteristic of beriberi heart disease.

De Wardener & Lennox (2) have confirmed the previous observation of Jolliffe *et al.* (3) in regard to Wernicke's encephalopathy. This type of encephalopathy appears to be produced by an acute insufficiency of thiamine. Of the fifty-two cases in the report of de Wardener & Lennox, thirty recovered. These authors suggest that acute thiamine deficiency is the sole cause of the syndrome.

Rafsky, Newman & Jolliffe (4) have been studying the relationship between gastric acidity and the secretion of thiamine in the aged. Forty-five per cent of their series of thirty-one elderly subjects had low thiamine excretory levels. They found both high and low excretory levels of thiamine in their subjects with anacidity, hypochlorhydria, and hyperchlorhydria.

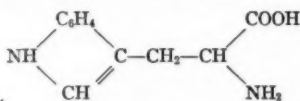


FIG. 3.—TRYPTOPHANE

NICOTINIC ACID

The physician may diagnose pellagra either in the adult or in the child by identifying typical skin lesions or characteristic mucous membrane lesions, or both. The skin lesions may appear on any part of the body. One sees them most frequently over sites of irritation such as the backs of the hands, wrists, elbows, knees, feet, and face. The areas of dermatitis are never static. They either advance or regress. In the early stages they resemble sunburn. Later, the skin appears thick and roughened. Finally, after many recurrences, it becomes thin and atrophic. Glossitis and stomatitis

are common symptoms of pellagra, and mental symptoms as a part of the pellagrous syndrome have been emphasized for many years.

The occurrence of a "pellagrenic" agent in corn has been discussed by Woolley (5), by Krehl *et al.* (6), and by many others. The time-honored association of the occurrence of pellagra with the eating of corn most likely is due to the fact that persons whose diets consist largely of corn have a deficiency of nicotinic acid (Fig. 2) and tryptophane (Fig. 3). Spies (7) has always recommended as an ideal diet for combatting pellagra one that is high in protein, vitamins, and minerals, supplemented by the indicated vitamins. It is unfortunate for the people who eat large amounts of corn that it happens to be low in both nicotinic acid and tryptophane. However, many diets other than those consisting largely of corn can produce pellagra. That corn is not essential to the pathogenesis of pellagra has long been known and the disease has been described in epidemic proportions among the inhabitants of some of the Japanese prison camps in the recent war. These prisoners lived on a diet low in calories, protein, and sources of the B complex. Their diet was, on a percentage basis, high in carbohydrate, and entirely lacked animal protein. The pellagra among these persons, as elsewhere in the world, was complicated by other clinical syndromes. There are many other unsolved problems of nicotinic acid metabolism which have been discussed by Handler (8). As yet, no physiologic role has been ascribed to the unbound nicotinic acid. Good food sources for nicotinic acid are beef and hog liver, kidney, lean meats, chicken, yeast, peanuts, salmon, eggs, milk, coffee, and certain green vegetables.

The use of nicotinic acid in pellagra is well known. There is an association between clinical pellagra and certain macrocytic anemias which will be discussed more fully in the paragraphs concerning folic acid. As has been shown by Spies (9), there is a glossitis which may be associated either with pellagra or with nutritional macrocytic anemia and, as the term is used clinically, may be relieved temporarily by either folic acid or nicotinic acid. Nicotinic acid does not relieve the associated macrocytic anemia whereas folic acid does. Spies considers nicotinamide, not folic acid, as a specific therapeutic agent for pellagra. It is well to recognize that each nutrient may have some specificity and, in addition, a general function. This is another example of the interrelation between the nutrients.

RIBOFLAVIN

Early in the history of pellagra, many writers noted atypical forms of pellagra. By 1938, Sebrell & Butler (10) had shown that clinical pellagra might be associated with cheilosis. The next year, Spies *et al.* (11) reported ocular lesions in man which could occur as a result of a riboflavin deficiency. Riboflavin (Fig. 4) is essential for man and for most other animal species. Good food sources are liver, kidney, meats, milk, powdered whey, cheese, eggs, fish, yeast, whole grains, peanuts, peas, bananas, ice cream. Riboflavin plays a most significant role in biological respiration. Riboflavin deficiency in man may cause cheilosis and eye disturbances charac-

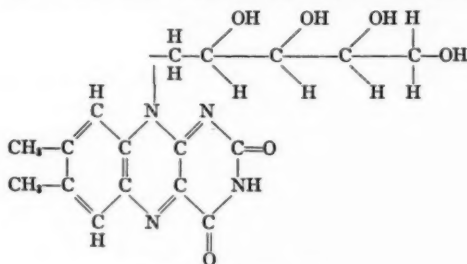


FIG. 4.—RIBOFLAVIN

terized by itching, burning, sensitivity to light, and vascularization of certain parts of the eye. In various parts of the world both cheilosis and ocular lesions are now found in persons subsisting on diets low in riboflavin. Yudkin (12) presents evidence that about one fifth of the army personnel in Sierra Leone consumed a diet deficient in riboflavin. The men developed positive signs of riboflavin deficiency which were relieved by the administration of riboflavin. It is inconceivable to the reviewer that they had a deficiency of riboflavin alone, for in his experience this deficiency is associated with a deficiency of other water-soluble vitamins as well as certain of the fat-soluble vitamins. Cheilosis may be caused by many medical conditions other than riboflavin deficiency, and a careful differential diagnosis is always necessary. It is unfortunate that some physicians have regarded cheilosis as pathognomonic of riboflavin deficiency. Rest in bed or relief for insomnia or remedies directed toward improving other symptoms often improve the general condition and the lesions decrease or disappear. The ocular

lesions are similar in that many agents which tend to increase the general health of the patient will relieve the ocular lesions. It seems obvious that a number of fundamental anatomical structures are involved and that a number of different factors can predispose toward or produce such lesions. It is good that much health skepticism has arisen among the clinicians. If one studies undernourished persons year after year, he finds that they complain periodically of weakness, tiredness, irritability, and many other vague symptoms which can be relieved by proper diet. The reviewer recommends that the nutritional status of all patients with ocular lesions or cheilosis be carefully studied and that riboflavin be administered if the person has any evidence of a deficiency in diet.

ASCORBIC ACID

The physiological functions of ascorbic acid (Fig. 5) are many. The most clearly defined function is the formation of reticulum and collagen so as to maintain the integrity of the intercellular substance. Bone undergoes constant change. The collagen of bone, like collagen elsewhere in the body, is influenced by many factors.

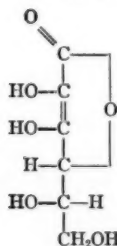


FIG. 5.—L-ASCORBIC ACID

Failure of adjustment of collagen appears to be one of the consequences of older age. Vitamin C is necessary for the synthesis of the protein constituent of collagen. Ascorbic acid deficiency results in inhibiting bone formation not by interfering with the inevitable cartilage sequences but by preventing the laying down of collagenous bone matrix. The scorbutic lesions of the gums are difficult to explain. The gingiva is involved only when teeth are present. The hemorrhages all over the body and especially over the skin may follow stress. Certainly studies of the integrity of the capillary have shown that morphological changes are wanting. Scurvy and

anemia do not necessarily coexist, yet Vilter *et al.* (13) have shown that severe scurvy may be associated with a macrocytic anemia which is not corrected as long as the patient is on a diet restricted in vitamin C but that it is corrected by the addition of vitamin C even if the patient continues to eat a diet restricted in vitamin C. The recent studies of Harris *et al.* (14) indicate that the problem of diagnosing subclinical deficiencies remains perhaps the most important on the medical agenda of scientists today. Laboratory methods today are not as satisfactory as could be wished. Most patients have months or years of ill health before a satisfactory diagnosis is made. These studies of Harris and his associates conclusively show that mixed deficiency diseases are of common occurrence in persons subsisting on deficient diets. Basinski & Seacock (15) have shown that phenylpyruvic acid and L-tyrosine are compounds exhibiting an ascorbic acid-dependent metabolism.

FOLIC ACID

Although folic acid is the newest vitamin known to be of importance in human nutrition, many investigations of its clinical value already have been made. There is abundant evidence to show that it may be effective when there is failure of bone marrow regeneration and peripheral hemocytopenia whereas thiamine, nicotinic acid, riboflavin, pantothenic acid, pyridoxine, inositol, *p*-aminobenzoic acid, choline, or any of the other known vitamins are not effective in these conditions. Folic acid, which is chemically called pteroylglutamic acid (Fig. 6), leads to a favorable blood response in persons with Addisonian pernicious anemia, nutritional macrocytic anemia and in the macrocytic anemia of sprue, pellagra, and pregnancy. It is nontoxic when given in several thousand times the therapeutic dose. In persons who show hypersensitivity to various liver extracts, folic acid may be substituted safely for liver extract to induce blood regeneration. Frommeyer *et al.* (16) have shown that the clinical and hematologic response which follows the administration of folic acid is somewhat comparable with that which follows liver extract when the patients have comparable blood values. Zuelzer (17), in discussing the pathogenesis of anemia in infancy and childhood, notes the importance of folic acid in stimulating hemopoiesis in megaloblastic anemias. The chemistry of the antianemic substance present in yeast and liver is still unknown. That liver extract contains an active material other

than folic acid which protects against the combined system disease that so frequently develops in persons with Addisonian pernicious anemia has been amply demonstrated by Spies *et al.* (18), Vilter *et al.* (19), Meyer (20), and Heinle *et al.* (21). The studies of Vilter *et al.* present evidence that folic acid not only failed to prevent the development of combined system disease but that large doses did not halt the process once it had begun. The full benefit of folic acid in the treatment of pernicious anemia must await the discovery of means of protecting against degeneration of the nervous system.

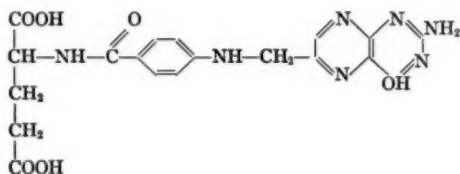


FIG. 6.—LIVER *L. casei* FACTOR (PTEROYLGLUTAMIC ACID)

The reviewer has not seen any signs of degeneration of the nervous system in any typical case of nutritional macrocytic anemia or of tropical sprue. Garcia Lopez *et al.* (22) have reported on the rehabilitation of eighteen persons with tropical sprue following folic acid therapy. In each of these eighteen cases there was a prompt regeneration of the blood and a tendency of the alimentary tract to return to normal function. Despite the fact that antiparasitic therapy was not administered, the presence of pathogenic intestinal parasites did not prevent these persons from being profoundly benefited. Examination of these patients two months after therapy was discontinued revealed that each of the five women in the study had returned to their full household duties and that nine of the thirteen men had returned to work. Of the four men who did not return to work, one was a cripple who had never been able to work and the other three, who were seventy-five years of age, had retired. Following therapy, however, these four persons were able to care for themselves, whereas prior to treatment they had been bedridden and dependent on their families or friends for their personal care. The studies of Suarez *et al.* (23) have amplified greatly the value of folic acid in the treatment of tropical sprue. They have learned that a small dose of 10 mg. or so administered daily is more effective than a much larger dose given only once.

Studies of Spies *et al.* (24) have shown that the folic acid molecule is very specific and must be prefabricated in order to be effective in producing a hemopoietic response in human beings. Pteric acid (Fig. 7) and glutamic acid (Fig. 8), whether administered separately or together, are ineffective. Aspartic acid (Fig. 9) and

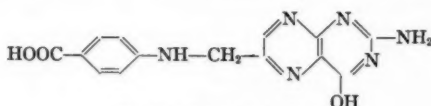


FIG. 7.—PTEROIC ACID

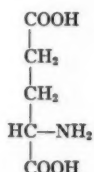


FIG. 8.—GLUTAMIC ACID

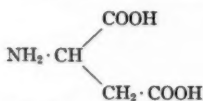
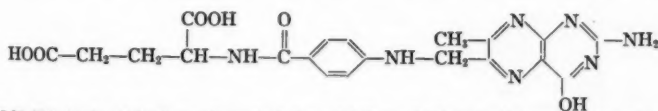


FIG. 9.—ASPARTIC ACID

glutamic acid are homologous amino acids yet aspartic acid cannot substitute for glutamic acid in the folic acid molecule. Spies and



N-[4-[(2-Amino-4-hydroxy-7-methyl-6 pteridyl) methyl] amino] benzoyl glutamic Acid

FIG. 10.—METHYL *L.* *Casei* FACTOR^a

co-workers (25) further demonstrated the specificity of the folic acid molecule in a study which showed that the addition of the methyl group (Fig. 10) makes folic acid an ineffective compound

^a The formula as shown is probably correct. As can be seen, the methyl group is attached to the 7 position but it might be attached to the methylene bridge and it may well be that the product used was a mixture of the two.

in stimulating blood regeneration. Spies *et al.* (26) have shown that the conjugated forms of folic acid, when given in adequate amounts, are effective in treating persons with tropical sprue, Addisonian pernicious anemia, and nutritional macrocytic anemia. Lewisohn *et al.* (27) have reported that eleven of twenty-eight

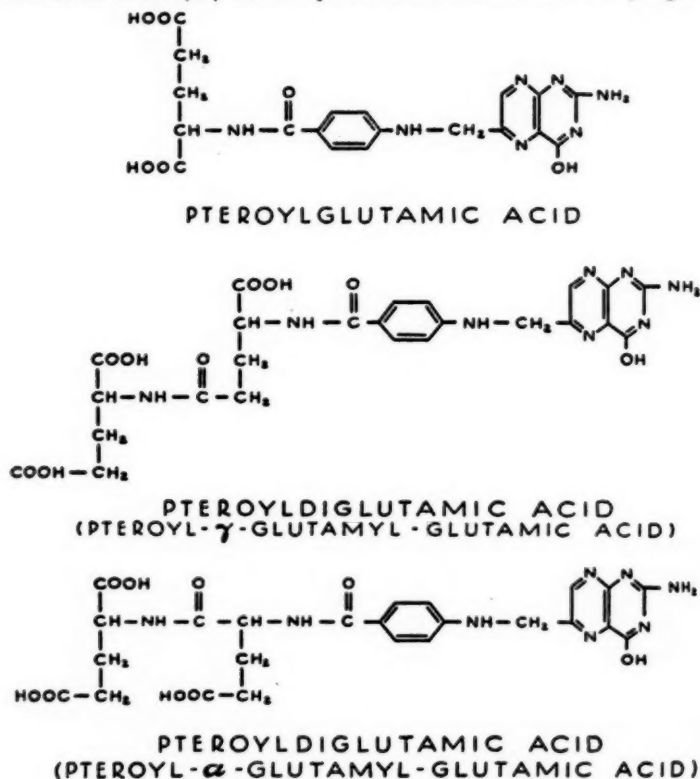


FIG. 11

mice receiving fermentation *L. casei* factor, which is chemically termed pteroyltriglutamic acid (Figs. 11, 12), showed complete regression of their primary tumors. According to newspaper reports, these authors have extended this observation to human beings. It is difficult, of course, to modify the progress of a tumor once it has

formed except by the use of agents which actually destroy cells. This reviewer has seen this substance given to patients with tumors but at the dosage level used and under the conditions observed, it was not effective.

In recent months "Diopterin" and "Teropterin" have been used by various clinical groups for the treatment of malignancies. As can be seen from Fig. 11, there are two pteroyldiglutamic acids. Pteroyl- α -glutamylglutamic acid ("Pteroyldiglutamic acid") has been synthesized by the Lederle Laboratories and goes under the trade name of "Diopterin." It is inactive for *L. casei* and

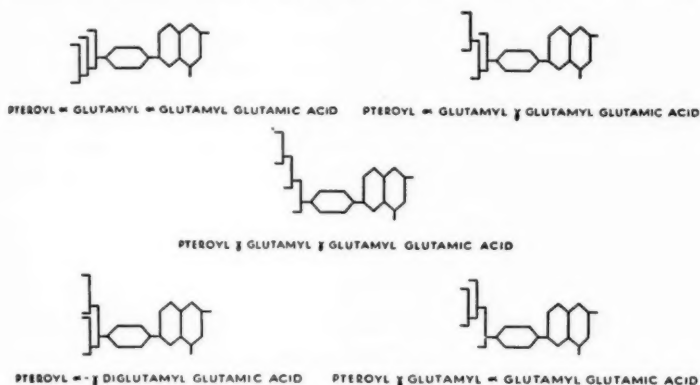


FIG. 12.—5 PTEROYLTRIGLUTAMIC ACIDS

S. faecalis R. but active in humans to produce a remission of macrocytic anemia with megaloblastic arrest of the bone marrow. It is said to relieve pain due to tumors but it does not cure the malignancy per se and apparently is excreted as pteroylglutamic acid. As far as the reviewer knows, it is not found free in nature.

Pteroyl- γ -glutamylglutamic acid has also been synthesized by the Lederle Laboratories and will produce a remission in certain anemias, but it has not been studied as fully as the α form.

According to Dr. Thomas H. Jukes, five pteroyltriglutamic acids exist. The abbreviated formulae are as shown in Fig. 12.

The Lederle Laboratories have synthesized pteroyl- γ -glutamyl- γ -glutamylglutamic acid, and it goes under the trade

name of "Teropterin." This compound is said to have some activity in inhibiting mammary tumors in mice. It is also said to relieve or decrease the pain arising from tumors in human beings but it can, in no sense, be regarded as a cure. It is active in producing remissions in certain anemias in man. It is relatively inactive for *S. faecalis* R. but active for *L. casei* and animals, including man. It was isolated from *Corynebacterium* fermentation residue.

CHOLINE

Copeland & Salmon (28) observed that neoplasms occurred in 58 per cent of rats kept on a choline deficient diet for eight months or longer, whereas not a single neoplasm developed in litter-mate controls receiving the same diet supplemented with 20 mg. of choline (Fig. 13) per rat daily. This very important study is concerned not with relieving tumors once they have developed but in preventing them in a susceptible strain. Clinicians are well aware that cirrhosis of the liver predisposes to primary tumors and that

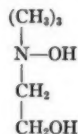


FIG. 13.—CHOLINE

choline deficiency is followed by cirrhosis of the liver. In this interesting experiment tumors of the lungs were observed. Did trauma to the lungs also occur? This must be interpreted in the light of cell physiology and adequate nutrition. Choline deficiency, if it exists at all in human beings, is not recognized as such. There must be an intensification of the study of the cancer problem. Such intensification can be directed along many lines. We already know that there is an association between various forms of industrial cancer, and that experimental cancer can be produced by chemical compounds. We must know more of the mechanism of the carcinogenic action of all the factors promoting and retarding the production of tumors.

DICOUMAROL

Allen (29) in his discussion of the new and ever more important subject of the clinical use of anticoagulants, states:

If blood did not possess the quality of coagulation, injuries would allow it to leak from the body as freely as water leaks through a sieve. This knowledge, which is possessed by every physician, has dominated medical thinking about coagulation of the blood. A vast proportion of the efforts of many physicians interested in coagulation has been devoted to making blood clot better, because of observation of the consequences of blood which clots poorly. There are few physicians who have not wished for an efficient method by which they might stop bleeding. It is quite clear now that thoughts of the medical profession must be directed in the reverse direction as well; in some circumstances there are great benefits if the property of coagulation of blood is impaired. When the vascular system of the blood is intact, coagulability of the blood may be reduced substantially without harm, and indeed with great benefit. It is doubtless true that hemorrhage causes far fewer deaths than intravascular thrombosis. The problem of intravascular thrombosis is, therefore, more important than the problem of hemorrhage . . .

The importance of diseases of blood vessels would be greatly lessened if vascular disease did not provoke thrombosis and if blood would not clot inside living blood vessels. Phlebitis would be a benign disease if it did not cause venous thrombosis. Arteriosclerosis would have lesser importance if it did not cause coronary and cerebral thrombosis. The two chronic occlusive arterial diseases of the extremities, arteriosclerosis obliterans and thromboangiitis obliterans, would not cause any great impairment of the arterial circulation to the extremities if thrombosis were not a part of them. The curse of cardiac irregularities, coronary thrombosis, and congestive heart failure would be substantially smaller if mural thrombosis and embolism were not associated with them. There would be no pulmonary embolism or phlebothrombosis if blood would not clot inside blood vessels; the gangrene of trench foot and immersion foot would be avoided in many instances. These observations emphasize that the health of man would be greatly improved if intravascular thrombosis did not occur. That which is desired is a method of impairing coagulation so that intravascular coagulation will not occur while the protective mechanism of coagulation which prevents bleeding remains. The use of anticoagulants is a step in the direction of achieving the prevention of intravascular thrombosis.

Allen (29), Barker (30), Levan (31), and others stress that intravascular thrombosis usually can be prevented when anticoagulants are used expertly. In recent years two substitutes, heparin and dicoumarol, have been introduced to inhibit intravascular thrombosis. Heparin is not a vitamin and will not be further discussed in this review save to say that, as an anticoagulant, it has certain advantages and disadvantages. The advantages are that it produces its effect rapidly, within five to ten minutes after a single intravenous injection, and that its effect is terminated a few hours after discontinuing its administration. It has disadvantages which will not be gone into since this review is concerned primarily with the vitamins. The administration of dicoumarol [3,3'-methylenebis(4-hydroxycoumarin)] (Fig. 14) produces prothrombin de-

iciency which can be measured by the prolongation of the Quick prothrombin time. Dicoumarol is effective when administered orally. (A satisfactory preparation for injection has not been developed.) Its effect persists for from one to two days after administration is discontinued. Barker, Levan, and Allen independently agree that the administration of dicoumarol requires daily careful ad-

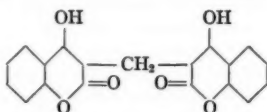


FIG. 14.—3,3'-METHYLENEBIS(4-HYDROXYCOUMARIN)

ministration and individualization of dosage. It is not recommended if facilities for the determination of accurate prothrombin time are not available. Hemorrhage due to prothrombin deficiency is the only toxic effect of dicoumarol and it can be controlled by fresh blood or vitamin K. Like many other chemical substances it may produce allergy in some individuals, characterized by urticaria and headache. Its use under controlled conditions should be considered in postoperative thrombosis and embolism, acute arterial occlusion of the extremities or the coronary artery. It is contraindicated in persons with renal insufficiency, liver damage, bacterial endocarditis, purpura, bleeding tendencies, and recent brain and cord injections or operations. Relative contraindications are peptic ulcers, open wounds, faulty absorption of vitamin K, and general emaciation.

p-AMINO BENZOIC ACID

Flinn *et al.* (32) have given large amounts of *p*-aminobenzoic acid (Fig. 15) to nine patients with Rocky Mountain spotted fever



FIG. 15.—*p*-AMINO BENZOIC ACID

who were under forty years of age. All of these patients recovered. In contrast, two of thirteen patients under forty years of age in a control series died. The number of days of fever in the treated cases was reduced about 50 per cent. In the treated cases there was

a decided clinical improvement preceding a drop in temperature and the temperature fell to normal within two to four days after treatment was started. After the temperature began to fall, the rash faded rapidly and the clinical recovery was uneventful. Further observations by Snyder *et al.* (33) on the treatment of typhus fever with *p*-aminobenzoic acid brought forth evidence showing that treatment begun early in the first week of illness was more effective than treatment begun late in the first week of illness. The observations of Pinkerton and his school and of Anigstein and Bader have been followed by the use of *p*-aminobenzoic acid in the treatment of several rickettsial diseases. Until recently, there was no special remedy for these diseases and even now it is difficult to persuade physicians of the necessity of giving large doses of this vitamin. Many physicians remonstrate when told to start with an initial dose of 8 gm., followed by 3 gm. every two hours until a blood level of 30 to 60 mg. per 100 cc. of blood is reached.

RUTIN

Rutin (Fig. 16), a flavonol glucoside derived from various plants including buckwheat, has been shown by Griffith, Couch & Lindauer (34) to decrease capillary fragility in man, when that

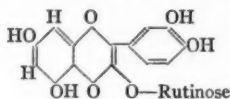


FIG. 16—RUTIN

fragility was originally increased. This role is distinct from vitamin C in protecting against capillary fragility and permeability. It has been suggested by Griffith *et al.* that rutin may be important in preventing certain vascular accidents which occur in persons with high blood pressure. The vitamin nature and behavior of rutin and the clinical indications are still to be established.

VITAMIN A

The lack of vitamin A (Fig. 17) in human beings is associated eventually with night blindness, keratomalacia, or xerophthalmia. In deficiency of vitamin A, certain specific pathologic changes are observed in the epithelial structures. Eventually they give rise to what can be termed vitamin A deficiency. This may be character-

ized by the loss of visual acuity in dim light, and within the past few years several groups of investigators have reported patients who had cutaneous lesions. Diabetic patients were found by Kimble *et al.* (35) to have levels of carotene and vitamin A outside the range of those established for healthy persons. Wolbach (36) has discussed in great detail the relation of vitamin A to skeletal growth. He points out that bone undergoes constant remodeling and that vitamin A lack or excess can affect bone growth directly. A deficiency of vitamin A causes all cartilage sequences to cease, whereas an excess of vitamin A administered causes an acceleration of all the normal cartilage sequences. It has been pointed out by many people, including Bakwin (37), that vitamin A deficiency is

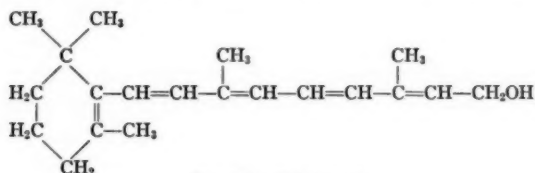


FIG. 17.—VITAMIN A

seen in infants with cystic fibrosis of the pancreas. Aron *et al.* (38) have studied the effect of elevated body temperature on plasma vitamin A and carotene. Both the plasma of vitamin A and carotene levels decrease with elevation of body temperature but return to normal after the fever disappears.

Vitamin A is found in fish liver oils, liver, egg yolk, cream, ice cream, milk, butter, and fortified margarines. Carotene occurs chiefly in the green leafy plants.

The physiologic effect of vitamin A is brought about in man by a number of different naturally occurring and synthetic compounds known as carotenoids. The carotenoids or carotenes occur in plants and are converted to vitamin A in the body. Vitamin A in large doses may be toxic. Arctic explorers who eat polar bear livers have developed toxicity to it which includes gastrointestinal symptoms and dermatitis.

VITAMIN D

The group of vitamins D contains a number of sterol derivatives which occur in nature chiefly in the animal organism. They can be produced by irradiating the corresponding precursors with

long ultraviolet radiation. The physiologic properties are similar but not identical. The two most important vitamins D for nutrition of mankind are vitamin D₂ (activated ergosterol, calciferol, viosterol) (Fig. 18), and vitamin D₃ (activated 7-dehydrocholesterol) (Fig. 19). The action of vitamin D in man is to increase the absorption of calcium and phosphorus of the food.

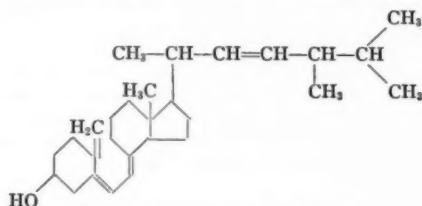


FIG. 18.—CALCIFEROL (VITAMIN D₂)

A deficiency of vitamin D results in the development of rickets in infants and children and of osteomalacia in adults. In rickets, there is a failure of calcification in the bony matrix and also the mature cartilage cells fail to degenerate. Barnes *et al.* (39) have studied the relative effect of viosterol, tuna liver oil, and cod liver

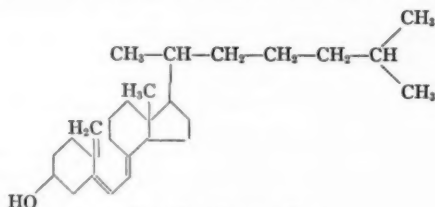


FIG. 19.—VITAMIN D₃

oil as sources of vitamin D. The phosphatase level which is known to be high in rickets was used as the criterion of recovery. They did metabolic studies on thirty infants and noted that a 20,000 unit dose of cod liver oil concentrate produced a greater rise in phosphorus retention than had been expected and that smaller concentrations of cod liver oil, tuna liver oil, and viosterol produced rises which, although definite, were smaller than expected. Their data emphasized in general the difficulty in judging the rachitic state from phosphorus values alone. It is well known that there are many distinct antirachitic vitamins and that some have

more effect in preventing rickets in some species than in others. The reviewer has observed that the patient with rickets must be treated adequately by clinical and laboratory methods and should be treated longer than is usually the case. Rickets is still sufficiently common to demand the active interest of all physicians in a campaign against it. Follis *et al.* (40) have made a careful study which shows a startlingly high incidence of rickets in children from two to fourteen years of age. These authors conclude:

We doubt if slight degrees of rickets, such as we found in many of our children, interfere with health and development, but our studies as a whole afford reason to prolong administration of vitamin D to the age limit of our study, the fourteenth year, and especially indicate the necessity to suspect and to take the necessary measures to guard against rickets in sick children.

An overdose of vitamin D may lead to the calcification of the soft tissues and even death. Bauer *et al.* (41) have pointed out, as have many other investigators, an abnormal calcium deposition following vitamin D intoxication. These authors state:

Mild symptoms of vitamin D intoxication, such as gastrointestinal and genitourinary complaints, are not uncommon during the use of large doses of this drug. It is important to detect these earlier symptoms and reduce the dose or withdraw the drug to avoid the potential danger of more serious toxic effects. The most severe toxic effect is the occurrence of metastatic calcification, which may lead to death. This is rare in the human being and especially in the adult, but the fact that it does occur should suffice to make one cautious. The fact that reported deaths from excessive amounts of vitamin D have usually been in infants may suggest greater susceptibility of the young or simply that the doses given were greater per unit of body weight. However, until recently large doses of vitamin D have been given only to infants and children, and in most adults the symptoms of vitamin D toxicity are appreciated and the drugs discontinued, whereas in infants its administration may be unwittingly continued.

In recent years large doses of vitamin D preparations have been prescribed for the treatment of a wide variety of diseases, including arthritis, pollinosis, tetany, psoriasis, acne, and trichonosis. A discussion of the efficacy of this form of therapy in these widely divergent clinical conditions is beyond the scope of this paper, but it might be well to state that in most cases the administration has been largely on an empirical basis and the results reported vary considerably, many being contradictory. It is regrettable that the use of these potentially toxic preparations has been advocated in the lay press and the medications have been sold to the public without need for prescription. Our case is an example of the injudicious self medication with one of these preparations and indicates their potential danger. There is great variation in species susceptibility and individual susceptibility to the toxic action of vitamin D. It is usually stated that the threshold toxicity in the human being is 20,000 international units per kilogram of body weight daily, but Reed and his associates report toxic effects in one adult with as

little as 1,000 international units per kilogram of body weight daily. The severe toxicity in our patient was produced by from 2,200 to 11,000 international units per kilogram daily. The susceptibility of an individual will vary at different times, and Reed, Struck and Steck found that toxicity was more likely to occur in patients with gastrointestinal dysfunction, especially constipation and diarrhea. They reproduced toxicity at will by creating a gastrointestinal disturbance in a person taking large but previously subtoxic doses of vitamin D. According to these investigators the factors which influence the toxicity of vitamin D are the dose per unit of body weight, the route and duration of administration, the composition of the diet in regard to the mineral and organic contents, the state of the alimentary tract, species susceptibility, age, pre-existing pathologic conditions, the vehicle in which the vitamin is administered, the state of the endocrine system, the purity of the vitamin preparation, the source of the vitamin, and other factors not yet recognized.

VITAMIN K

Vitamin K (Fig. 20) is essential for the formation in the body of prothrombin. Prothrombin plays a major role in the process of blood clotting. A deficiency of vitamin K results in a prolongation

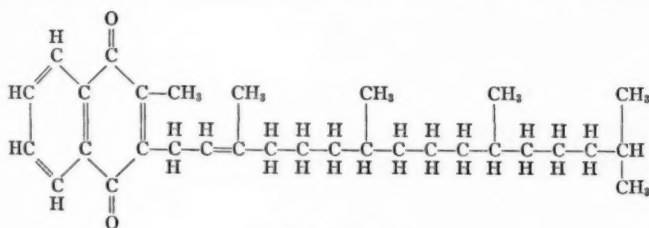


FIG. 20.—VITAMIN K

of the normal clotting time of the blood. It is found in hemorrhagic diseases of the newborn, diseases of the biliary tract, and certain intestinal disorders. Vitamin K prophylaxis for the newborn is now routine in the practice of nearly all obstetricians. The vitamin K can be administered to the mother shortly before the child is born or to the newborn infant itself.

It is interesting that vitamin K ("menadione"—2-methyl-1,4-naphthoquinone) (Fig. 21) refers to a group of substances of the naphthoquinone class. "Menadione" is the standard for expressing potency of the various ones of the vitamin K group. Vitamin K is required by all animals but the daily requirements are probably supplied in adequate amounts by intestinal synthesis. The natural vitamin K can be found in alfalfa, kale, spinach, dried carrot tops,

tomatoes, chestnuts, soybean oil, putrefied fish meal, bran, casein, and other sources.

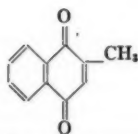


FIG. 21.—2-METHYL-1,4-NAPHTHOQUINONE

TOCOPHEROLS

Vitamin E is the term used to refer to a group of substances having similar actions. These substances are α -, β -, γ -, and δ -tocopherol. α -Tocopherol (Fig. 22) is the most potent and is used for the standard of reference. The clinical function of vitamin E is a subject of great controversy. Its significance in human nutrition has not as yet been conclusively established. Vogelsang *et al.* (42) have recently reported that anginal pain is greatly reduced and that the clinical edema associated with heart failure disappears following its use. These studies demand more investigation. Shute (43) has reported that it is effective in three cases of acute nephri-

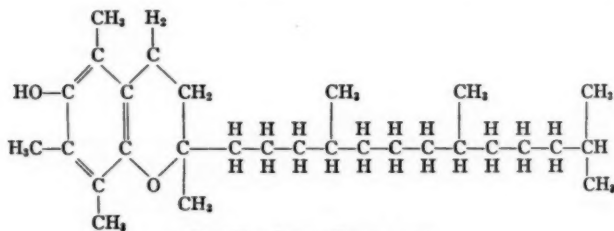


FIG. 22.—ALPHA-TOCOPHEROL

tis. Again there is not enough evidence to warrant recommending the substance for routine use in acute nephritis. Steinberg (44) gave an average daily dose of 300 mg. of mixed natural tocopherols to persons with fibrositis. He obtained some clinical response within two to four weeks. He treated Dupuytren's contracture with relief in a number of instances. The blood levels of a large group of people with other deficiency diseases were found to be low in tocopherol by Harris *et al.* (14).

Goldsmith (45) in a recent summary of the importance of vita-

mins of the B complex in clinical medicine, has related the use of the B vitamins to the requirements. This article should be read in its entirety.

Many of the vitamin deficiencies are secondary to other organic diseases and the recent paper by Sevringhaus (46), in which he discusses the role of vitamin therapy in the management of diabetes mellitus, is relative to most secondary vitamin deficiencies.

In reviewing accomplishments in the clinical applications of vitamins for the past two years, various individuals will differ in their decisions on what to include. While we have placed emphasis on the treatment of various deficiency states in the limited space at our disposal, we have discussed more of the broad fundamental advances. It would take several volumes to treat the subject exhaustively.

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THE BIOCHEMISTRY OF CARCINOGENESIS

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The last general review on this subject in the *Annual Review of Biochemistry* was published in 1945. In 1947 two extensive reviews appeared: Greenstein's monograph on the "Biochemistry of Cancer" and the review on "Chemical Carcinogenesis" in the *British Medical Bulletin*. Only the most recent papers are included in the present review and because of limitation in space, investigations on viruses, immunology, and chemotherapy have been omitted.

THE INDUCTION OF TUMORS

Pure chemicals.—The most recent work on the carcinogenic azo dyes has been reported by the groups at Wisconsin (1 to 4) and at the Memorial Hospital, New York (5). The most extensive investigation was made by Miller & Miller (6) who tested the carcinogenic activities of forty compounds that were either structurally similar to or were possible metabolites of *p*-dimethylaminoazobenzene. They suggested that two conditions appear to be essential if an aminoazo dye is to have high activity: (a) at least one methyl group must be attached to the amino group together with the proper second substituent, and (b) the rings must bear either no substituents or carry only certain substituents in the 3' position. The results of these studies indicate that the primary carcinogen operative in tumor formation by *p*-dimethylaminoazobenzene is probably an azo dye closely related to the parent dye.

Of the agents capable of producing neoplasms in various tissues, 2-acetylaminofluorene, 2-aminofluorene, and related compounds (7, 8, 9) have recently received considerable attention. When these agents were incorporated in the diets of rats neoplasms were observed in the ear duct, eyelid, breast, lung, liver, bladder, salivary glands, gastrointestinal tract, and nerve tissue (9 to 14). 2-Acetylaminofluorene incorporated in the diet has been found to be carcinogenic for three species beside the rat, viz., the mouse (15), the fowl (16), and the cat (9). No neoplasms of the thyroid or pituitary were observed in rats that received 2-acetylaminofluorene alone. When it was administered with allylthiourea (17),

adenomas of the thyroid were produced, and when given with stilbestrol, malignant tumors of the pituitary were observed (9). However, the administration of thiourea, by itself, has been shown to induce tumors of the thyroid (18).

A new class of carcinogenic substances, 4-aminostilbene and its derivatives, has recently been studied (19). The aminostilbenes show a considerable structural similarity to the aminoazo dyes, but as carcinogens they more closely resemble the aminofluorenes. The tumors produced in the rat include sarcomas at the point of injection, carcinomas of the eyelid and external auditory canal, subcutaneous fibromas, and neoplasms of the lung, liver, breast, and intestine. Like acetylaminofluorene these compounds have long latent periods (19).

Other chemicals of known structure recently shown to have carcinogenic activity include urethane, which induces neoplasms in the lungs of mice (20, 21) and rats (22). The inhibiting effect of urethane on mitosis has also been reported (23). An older observation on the carcinogenic action of Light green SF for rats has recently been confirmed (24).

Papers on hydrocarbons have been omitted since a recent extensive review of the subject is available in the *British Medical Bulletin* (19).

Carcinogens of biological origin.—Although most investigations with carcinogenic agents have involved studies of compounds of known chemical structure, the search for carcinogens of biological origin has also continued. Since 1920 a number of reports have appeared stating that sarcoma is an almost inevitable complication of infestation of the liver of rats with *Cysticercus fasciolaris*, the larva of the common tapeworm of the cat. Dunning & Curtis (25) demonstrated an active agent in the washed, ground *Taenia* larvae, which initiated multiple peritoneal sarcomata in fifty-one of fifty-six rats following its injection into the peritoneal cavity. The active agent was found to be associated with the calcium carbonate corpuscles of the parasite, and all experiments with desiccated, fractionated, filtered or frozen larvae have been negative in result. Smegma has also been tested for carcinogenic activity and although the subcutaneous or intravaginal injection into mice of human smegma caused no neoplasms (26), a few skin tumors were induced in mice at the site of injection when horse smegma was used (27). The presence of cancer of the mouth in natives of various

islands in the South Pacific has long been attributed to chewing of the Betel nut, but recent evidence does not substantiate this (28).

According to one theory of cancer, some tissues of the body produce carcinogenic agents by an aberration of those processes which synthesize the steroids. Such an explanation seemed simpler fifteen years ago than it does today, partly because, since that time, the number and variety of known carcinogenic compounds has increased greatly. However, a number of investigators have extracted human and animal tissues in a search for carcinogenic substances and have observed varying degrees of activity. Shabad (29) and Steiner *et al.* (30) noted active agents in the lipid non-saponifiable fractions of liver. Hieger (31, 32) has obtained a cholesterol-rich fraction from tissues of human subjects that produced some sarcomas following injection into mice. A carcinogenic substance was also found in the lipid fraction of the pituitary of cattle (33).

Carcinogenic factors of dietary origin.—A number of investigators have demonstrated that heated fats have a low degree of carcinogenic activity; the most ardent exponents of this idea during the past few years were Peacock and his associates (34). Black crusts of bread have also been incriminated as a source of carcinogenic substances, but the experimental evidence for this is inconclusive (35).

Another lead has appeared in a publication by Copeland & Salmon (36). In a strain of hooded rats a chronic choline deficiency was developed from the time of weaning, and of the fifty surviving over eight months, 58 per cent developed one or more types of neoplasms, the most common sites being the liver and lungs. No neoplasms were found in control animals receiving 20 mg. of choline chloride daily. A more thorough study of the pathogenesis of these tumors is indicated. The formation of neurofibromas of the ears of the rat by the prolonged feeding of 5 per cent of crude ergot was reported by Nelson and his associates (37). The tumors regressed upon withholding the ergot, but reappeared on refeeding. In a few old rats tumors recurred even though ergot had been withheld for six months.

Radiant energy.—The new information in the field of radioactive carcinogens includes work done by exposing animals to the radiations of plutonium and various radioactive products of uranium fission. Brues, Lisco & Finkel (38) found that bone sar-

comas were produced by feeding strontium (Sr^{90}), radioactive cerium-praseodymium ($\text{Ce}^{144}\text{Pr}^{144}$) and plutonium (Pu^{239}). Sarcomas of the bone also were observed following the ingestion of radium (39). Fibro-sarcomas at the site of injection also appeared with the latter element. Neoplasms of the large intestine occurred in rats fed yttrium (38). The carcinogenic effect in rats and mice of high-energy radiations associated with the uranium chain reaction was described by Henshaw *et al.* (40). Penetrating radiations resulted in generalized atrophy and neoplasms of the hemopoietic tissues while the less penetrating radiation caused neoplasms of the skin in a large percentage of the animals. The pathologic effect of high levels of instantaneous radiations on humans in Nagasaki and Hiroshima was described by Warren (41) and it is possible that latent effects of such radiation may result in leukemia or ovarian tumors similar to those induced in mice following x-ray (42). Miszurski *et al.* (43) have shown that doses of x-rays that were lethal to slow-growing nontransmissible Rous fowl sarcoma cells were harmless to the causative agent and influenced the tumors in such a manner that some of them became transmissible. The production of showers of rays under lead plates by cosmic radiation has been reported by Figge (44) to increase the incidence of neoplasms in mice injected with methylcholanthrene. The use of a filter that transmits only the 2900 to 3300 Å bands has localized more precisely the carcinogenic wavelengths of ultraviolet radiation (45). The effect of temperature on the rate of tumor formation by these rays has also been studied (46).

Transplantable tumors.—Investigations on the heterologous transplantation of tumors into the anterior chamber of the eye of alien species have continued (47, 48) and Sano (49) reported that the preliminary passage of human lymphosarcoma in tissue culture permitted subsequent growth when transplanted into mice. The transformation of embryonic tissues into neoplastic cells following the impregnation of such tissues with methylcholanthrene prior to their transplantation into hosts of the same strain has been reported (50, 51). When such tissues as the lung, stomach, intestine, skin, muscle and cartilage were treated in this way before inoculation some tumors were observed in the very brief latent period of from 20 to 40 days. Neoplasms of the prostate or lung were also obtained with adult tissues by the same technique (52, 53).

METABOLISM OF CARCINOGENS

A detailed study of the metabolism of the hepatic carcinogen *p*-dimethylaminoazobenzene has been made by the Millers (6, 54 to 60). One of the initial steps is the reversible demethylation of *p*-dimethylaminoazobenzene to *p*-monomethylaminoazobenzene, which has the same carcinogenic activity, and an irreversible demethylation to *p*-aminoazobenzene, an essentially inactive compound. Many other N-substituted aminoazo dyes are similarly dealkylated to primary aminoazo compounds (6, 61). Low levels of all three aminoazo compounds are found in the liver and a larger amount of aminoazobenzene is carried by the red blood cells. About 40 per cent of the dye administered is excreted in the urine as conjugates of *p*-aminophenol and *p*-phenylene diamine (57). Smaller quantities of three other monophenyl amines and the *o*'- and *p*'-hydroxy derivatives of *p*-aminoazobenzene and *p*-monomethylaminoazobenzene have also been detected in the urine. Of probably greater physiological significance is the formation of metabolites capable of combining with the liver proteins *in vivo* (54).

A considerable amount of work has been done on the metabolism of the hydrocarbons. The usual metabolism involves the formation of a phenol (62, 63). The phenolic hydroxyl group is usually introduced into the α -position of the naphthalene nucleus or into analogous positions in more complex hydrocarbons. It is possible that these phenols are produced through the stages of "perhydroxylation" to a dihydroxy-dihydro-compound, and subsequent dehydration to the phenol. Weigert & Mottram (64) describe four different metabolites obtained from blue-fluorescent tissues of animals treated with benzpyrene. Some of these fluorescent compounds were easily extracted while others appeared to be more strongly absorbed by the cells of various tissues. Berenblum & Schoental (63) have identified the presence of 3,4-benzpyrene-5,8-quinone in the excreta of mice injected with 3,4-benzpyrene. When 3,4-benzpyrene is added to flasks containing autoxidizing linoleic acid, small amounts of 3,4-benzpyrene-5,8-quinone and 3,4-benzpyrene-5,10-quinone have also been found in this mixture (65). 3,4-Benzpyrene is a good antioxidant but is destroyed during the process, according to Mueller & Rusch (66). Thus the oxidations of benzpyrene *in vivo* and *in vitro* have a common product and possibly a common point of attack.

FACTORS THAT INFLUENCE CARCINOGENESIS

Agents applied with carcinogens.—Reviews on the effect of solvents on carcinogenesis have been presented by Rusch (67) and Dickens (68). The existing data suggest that oils from plant sources augment carcinogenesis while a number of lipids from animals retard it (69). Fats obtained from plant or animal sources differ in many respects, but one variation worthy of investigation is the greater stability of vegetable oils to autoxidation. Tests by Rusch *et al.* (69) on a number of lipids varying in their susceptibility to oxidation failed to give a clear answer to the problem although in some cases the addition of tocopherol decreased the rate of destruction of the carcinogen and favored the formation of tumors. Similar studies with hydrogenated lipids were nonconclusive (70). Since hydrocarbons are oxidized in the presence of oxidizing fats, the rate of disappearance of the carcinogen was determined but could not be correlated with the subsequent tumor incidence (69). Dickens (68) has found that the more rapid elimination of benzpyrene was associated with the higher carcinogenic activity, and slower elimination with lower activity, but there are exceptions to this finding (71).

Lanolin has long been known to retard the formation of skin cancer when applied together with the carcinogen. Since lanolin allays the irritating effect of the carcinogen, its anticarcinogenic influence may be due to some bland neutralizing action. However, the inhibiting effect of lanolin may be ascribed to its physical characteristics since its pasty consistency might permit only small amounts of the dissolved hydrocarbon to obtain contact with the surface of the skin and in effect would result in the application of subcarcinogenic doses (72). It is probable that no one theory will suffice to explain the differences in tumor formation when various agents are employed as solvents. Factors such as differences in the site of application, the amount of dispersal of the applied solution, or differences in tissue response, such as the amount of irritation, variation in the degree of encapsulation, vascularization, or phagocytic infiltration all must be considered.

Other agents that retard neoplasia include acid chlorides, bromobenzene, unsaturated acids, and the mercapturate-forming hydrocarbons naphthalene, anthracene and phenanthrene (73). These substances may alter sulfur metabolism and thereby oppose the mechanism of carcinogenic action (74). Taking a lead

from examples of competitive inhibition in the vitamin field Lacasagne and associates (75) observed a slight inhibition in tumor formation when methylcholanthrene or 1:2:5:6 dibenzanthracene were applied to the skin of mice together with structurally related but noncarcinogenic compounds. These results cannot be accepted as final until the experiment is repeated with an adequate number of mice; furthermore, no mutual influence of two different types of carcinogenic agents applied at the same time has been observed (76, 77).

Effect of diet on carcinogenesis.—From time to time accelerating or inhibiting effects on the formation and growth of tumors has been ascribed to various vitamins. An acceleration of cerebral tumors due to methylcholanthrene has been reported in rats kept on a diet deficient in thiamine (78). A slight retardation of the growth of mammary adenocarcinomas in mice following injections with thiamine or nicotinamide has also been reported (79). A pyridoxine deficiency had a slight but consistent retarding effect on the induction and growth of tumors in both mice and rats (80), whereas, rats in a state of vitamin A deficiency showed a high level of pyruvate in the blood and were more susceptible to carcinogenic substances (81). High levels of riboflavin afforded no protection against neoplasms induced with methylcholanthrene (82).

For a number of years Lewisohn and his associates have been testing the effects of several vitamins on the growth of mouse tumors. They have reported an inhibiting effect of inositol which was counteracted by *p*-aminobenzoic acid or pyridoxine (83). An inhibiting effect of avidin and desthiobiotin was observed when these were given separately, but a neutralizing influence was noted when they were administered together. The inhibiting effect of folic acid on several types of mouse tumors has also been reported by this group (84). The injection of 5 μ g. of folic acid daily resulted in complete regression of 43 per cent of the spontaneous mammary tumors. This finding was confirmed by the same group (85) with the extended finding that the "liver *L. casei* factor" gave the inhibition but that "fermentation *L. casei* factor" was completely ineffective.

A notable contribution in the field of anticarcinogenesis is the retarding effect of caloric intake on the genesis of tumors. According to Tannenbaum (86) the incidence and time of appearance of

tumors is correlated with the degree of calorie consumption. Rusch *et al.* (87) found a higher sustained blood glucose level in mice on high calorie diets than in the restricted control mice. However, the amount of glucose in the blood did not give a full answer to the problem, since the rate of tumor formation was also high in mice in which a considerable portion of the calories was supplied by fat although the blood glucose was in the normal range. Although the calorie effect is an outstanding phenomenon in itself, its significance in many aspects of the cancer problem has not been widely recognized. Too often investigators have ascribed a decrease in the development of tumors to specific factors, without first considering the influence of such factors on the caloric intake of the treated animals.

The number of factors that affect the formation of hepatic tumors induced by *p*-dimethylaminoazobenzene is considerable and places the formation of these neoplasms in a special category (88). Protection against tumor formation is obtained if the riboflavin content of the diet is raised to five times the level in the control diet (60). A similar protective effect has been obtained by replacing the corn oil in the diet by hydrogenated coconut oil, or its chief constituent, lauric acid (89). If the corn oil is left out of the diet, a synthetic detergent added (90), or casein replaced by egg white, tumor formation is likewise retarded (91, 92). Cystine added to the diet at a level of 1 per cent protects (93) but former reports that cystine deprivation also retarded tumor formation could not be substantiated with paired-feeding experiments (94). A variety of crude materials, such as dried liver, yeast, grain and milk have protective effects (88, 93). Factors that accelerate the formation of hepatic tumors include rice bran extract, high levels of fat, and low levels of riboflavin (60). In contrast to an earlier report (95) the most recent evidence indicates that the effect of biotin on the stimulation of hepatic tumors is demonstrable only under certain conditions and even then its effect is slight (96).

It appears that the level of riboflavin in the liver of the rat is an important factor in determining the probability that a given liver will develop a tumor when *p*-dimethylaminoazobenzene is fed, and it is of interest that the level of riboflavin was high in livers of rats fed protective diets and low in those on diets that stimulated tumor development (60). The riboflavin content of the liver can also be correlated with the carcinogenicity of certain azo

dyes: the most active carcinogens produced the greatest reduction in the level of this vitamin (97). The dietary factors that affect the development of tumors due to *p*-dimethylaminoazobenzene will not necessarily influence the formation of neoplasms due to other causes, i.e., the inhibiting effect of high riboflavin is present but not as pronounced when *m'*-methyl-*p*-dimethylaminoazobenzene or *o'*-methyl-*p*-dimethylaminoazobenzene are the carcinogens fed (4). Dietary factors also have less effect in rats on hepatic tumors induced by 2-acetylaminofluorene (98).

Effect of hormones on carcinogenesis.—A significant discovery concerning the influence of hormones on cancer is the observation that implantation of ovaries into the spleens of adult gonadectomized female rats is followed several months later by the development of tumor-like masses of granulosa cells (99). These experiments were based on two principles: (a) the ability of the liver to inactivate ovarian hormones when the hormones circulate through the hepatic portal system, and (b) the increase of pituitary gonadotropins subsequent to castration. The prolonged stimulation, by augmented amounts of gonadotrophic hormones, of intrasplenic ovarian grafts is believed to be responsible for the neoplastic growths. These observations have been confirmed and extended with mice by Li & Gardner (100). The latter investigators also observed that the development of ovarian tumors in intrasplenic ovarian grafts was inhibited by the administration of estradiol and testosterone (101). That splenic tissue does not play a direct role in the pathogenesis of ovarian tumors arising in grafts was demonstrated by the fact that ovarian tumors also occurred when intrapancreatic ovarian grafts were made (101).

The effect of steroid hormones on tumor formation is not limited to ovarian neoplasms. Estrogens are considered as potent leukemogenic agents (102) while androgens and adrenal cortical hormones retard leukemia (103, 104) as well as uterine tumors in guinea pigs (105). Gonadectomy of mice of the *ce* strain one to three days after birth resulted in a very high incidence of adrenal cortical carcinomas, but the formation of these neoplasms could be prevented by the administration of diethylstilbestrol (106, 107). Progesterone failed to inhibit the formation of mammary cancer in the Marsh-Buffalo strain of mice but resulted in a 32 per cent incidence of neoplasms at the site of injection (108).

Hepatoma formation due to 2-acetylaminofluorene is acceler-

ated by testosterone and estrogen administration (109) while estrogens stimulate and androgens inhibit the development of hepatic tumors and hemangioendotheliomas by *o*-aminoazotoluene in strain C. mice (110). The influence of sex hormones on the prostate gland has been reviewed by Huggins (111).

Gardner (112) noted the failure of testicular interstitial cell tumors to grow following transplantation into susceptible mice unless estrogens were also administered to the hosts. In the absence of an adequate hormonal environment the grafts regressed but small amounts of viable tumor persisted for periods up to seven months. Many of these persisting "rests" were so small that they could not be detected and failed to grow during periods of observation up to 312 days when the hosts received no estrogens, but the subsequent injection of estrogen resulted in the growth of these "rests." Once these tumors attained a certain size and were established in the estrogen-treated mice they continued to grow or persisted when treatment was discontinued for periods of 14 to 188 days (112).

MODE OF ACTION OF CARCINOGENS

The finding that certain carcinogens induce mutations has revived the old suggestion that cancer results from a somatic mutation. The induction of mutations in *Drosophila* with various chemical carcinogens (113), and the finding that the wave lengths that cause mutations in *Drosophila* (114) and maize (115) are identical with those that cause cancer, are examples of this point. Evidence by Strong (116) indicates that there is a correlation between the induction of germinal mutations by methylcholanthrene and the unknown changes in somatic tissue which lead to cancer induced by the same compound. An interesting theory concerning the energy relations underlying mutation has been suggested by Schrödinger (117).

The idea that the significant change in carcinogenesis is an alteration in protein has been suggested previously (74, 118), but the recent observation by Miller & Miller (54) that a firmly bound compound between proteins of the liver and an azo dye is found in rats fed *p*-dimethylaminoazobenzene is the first experimental evidence bearing on this point. They have shown that the bound dye is found only in liver tissue and only in the species of animals that develop hepatomas as a result of dye feeding. The amount of

bound dye is reduced when the level of riboflavin, a protective substance, in the diet is increased. The fact that no bound dye is found in the neoplasms of the liver is of even greater significance since this indicates that a qualitative difference in protein may exist between the liver and the tumor. The authors suggest that the combination of the carcinogen and an autotrophic protein could result in the gradual removal of this protein from the cell with or without the rise of other proteins. In any event *p*-dimethylaminoazobenzene might initiate the carcinogenic process through sublethal combinations of this dye or its metabolites with critical proteins in normal liver cells and their descendants. The autonomous tumor may be the eventual outcome of some of the damaged liver cells through a permanent alteration or loss of proteins essential for the control of growth but not for life (54).

Several attempts to explain the carcinogenic activity of chemicals on the basis of general molecular shape and dimension have been made recently (119). Others have concerned themselves with correlations of the energy states of the molecule and the carcinogenic activity, but not enough data are available at present to establish firmly such relationships as have been suggested (120, 121). Several investigators have proposed that it is not the hydrocarbon itself which is the proximate carcinogen, but rather the reactions involved, or a possible release of energy, during the transformation from one metabolite to another (62, 65).

The investigators who have considered a neoplastic cell as a variant or new race of cells have been listed by Haddow (122); the most recent suggestion along this line was made by Lederberg (123) on the basis of studies with mutant strains of *Neurospora*. Further evidence for a changed race of cells is obtained from experiments in which unicellular organisms were grown in the presence of carcinogens. Under such conditions, a small number of the cells have been shown to undergo an irreversible change and are then able to survive and multiply in an environment that made this difficult, or even impossible, for their parent cell (122, 124). The work of Earle (125) and of Firor & Gey (126) demonstrates that normal cells may also be transformed to malignant ones in tissue culture.

Any complete solution of the mode of action must make clear the relation between physical and chemical carcinogens and the tumor-producing viruses. The possible influence of radiant and

chemical carcinogens on certain protein constituents of the cell has been cited and recently several investigators have discussed the similarity of the viruses to certain constituents in the cytoplasm, i.e., microsomes, plasmagenes, etc. (122, 127, 128).

STAGES IN CARCINOGENESIS

Information is now also available on the possible stages in carcinogenesis. When subcarcinogenic levels of carcinogens were applied to the skin of mice, neoplasms were elicited in the skin by chemical, mechanical, or thermal irritation (129 to 133). Such irritation has been shown to be noncarcinogenic per se. These facts indicate that cancer formation occurs not as a continuous single process but rather as a series of biological changes. Furthermore, there are definite indications that carcinogenic chemicals are active only because of several distinct biologic properties and neoplasms seem to develop only as the result of the proper sequential summation of these properties (131, 132). Most investigators (129 to 133) agree that at least two stages are involved during carcinogenesis, and Kline & Rusch (131, 132) believe that enough evidence is available to justify the classification into three phases as follows: (a) Period of induction, during which the neoplastic cell is formed; (b) Critical period, a transitional period in which the growth of cells is in a delicate equilibrium with their environment and growth depends on the balance between the proliferative capacity of the cell and the local tissue resistance; and (c) Period of progression during which growth is relatively unchecked.

TISSUE CHANGES IN NEOPLASIA

Metals.—An integrated program for the study of changes occurring in mouse epidermis during methylcholanthrene carcinogenesis, first with special reference to metals, has been carried on for several years by the group at the Barnard Hospital. Carruthers & Suntzeff (134, 135) found that within ten days after painting mice with methylcholanthrene the calcium, iron, copper, and zinc contents of the resulting hyperplastic epidermis were markedly lowered from those of normal epidermis whereas sodium, potassium, and magnesium contents were little changed. The results were expressed with reference to nucleoprotein phosphorous. This new "equilibrium" remained for a period of several weeks until just prior to the time that squamous cell carcinomas could be detected.

In the resulting malignant tissues a further drop occurred in the same metal constituents. Confirmation of these findings on human epidermis and squamous cell carcinoma was also obtained (136, 137).

Other reports have appeared concerning the amount of these and other metals in normal and cancerous tissues. Decreases in manganese (138) and in iron and copper have been reported for human neoplasms (139). Brunschwig *et al.* (140, 141) noted less calcium and more potassium in malignant tumors of the stomach and colon of humans than in the surrounding normal tissues. Benign tumors had intermediate values.

An attempt to explain the significance of the reduced calcium content of malignant tumors was made by Coman (142) in data concerning the adhesiveness of cells. The lowered calcium content resulted in a reduced adhesiveness and a consequent better chance for migration to new sites in the body.

Vitamins.—Information on vitamin assays of tumor and normal tissues referred to in earlier reviews (143, 144) has been supplemented by several recent reports. Kirpichnikova (145) found low ascorbic acid levels in mammary glands of mice, an increase during pregnancy and very little in mammary tumors. Vitamin assays by Tatum *et al.* (146) on mouse epidermis during methylcholanthrene carcinogenesis revealed no significant changes in inositol, choline or *p*-aminobenzoic acid. Pyridoxine was increased after treatment with either the carcinogen solution or benzene alone while biotin was decreased significantly. According to Loo & Williams (147) the folic acid was altered in character in the livers of animals bearing transplantable tumors. Using two different buffers in clarase digestion for release of the liver folic acid, they found that normal liver released much more folic acid with sodium chloride-phosphate buffer than with acetate buffer, but this difference was not observed in animals with tumors. Vermes & Raffy (148) report low riboflavin content in human tumors but normal levels in the livers of cancer-bearing patients.

Nucleic acids and nucleoproteins.—Studies of nucleic acids have recently been facilitated by improvements in quantitative methods for their separation and identification. A report by Schneider (149) provides a rapid and simplified means of separating the nucleic acids from other phosphorus compounds in the tissues and permits measurement of very small quantities by micro-colori-

metric procedures involving the sugar components, ribose and desoxyribose. Schmidt & Thannhauser (150) describe the separation of the desoxyribonucleic and ribonucleic acids. A combination of features from these two reports (151) should be very helpful in tracer studies with radioactive phosphorus.

Considerable analytical data concerning the nucleic acid content of tissues have been reported. Schneider & Klug (152) determined the amount of both desoxyribonucleic and ribonucleic acids in a number of normal tissues and transplanted and primary tumors of the rat and found that the values for the tumors were among the highest. Khouvine & Grégoire (153) found a two- to threefold increase in the ribonucleic acid of human and rat tumors over the corresponding tissue of origin. Stowell & Cooper (154), using a photometric histochemical method, found less desoxyribonucleic acid in hyperplastic than in normal human epidermis and an increased content in epidermoid carcinoma. Davidson & Waymouth (155) noted a high level of total nucleic acid in fowl tumors and variable amounts in human tumors. Gopal-Ayengar & Cowdry (156) measured desoxyribonucleic acid in the chromosome threads during epidermal methylcholanthrene carcinogenesis in mice. They found a drop in desoxyribonucleic acid content of epidermal chromosomes, soon after application of the carcinogen, to a new "equilibrium" level. When tumors developed a rapid rise occurred. In a comparison between actively growing tumor tissue and necrotic areas (157), the ribonucleic acid was found to be low in the necrotic areas, but desoxyribonucleic was unchanged or higher. Analyses for nucleotides, nucleosides, uric acid, allantoin and urea in the blood gave no clue as to the identity of the degradation products.

Euler *et al.* (158) isolated nucleoproteins from rat liver and tumor and found no difference between them as enzyme substrates. However, the blood serum of normal rats was shown to have nucleic acid-splitting activity which was not demonstrable in serum from tumor-bearing rats.

Some attention has been given to the study of the effects of x-rays on nucleic acids and nucleoproteins. Irradiation of nucleic acid with x-rays reduced its viscosity but did not modify its activity as an enzyme substrate (159). Errera (160) found that nucleoprotein gels were liquified by x-rays. The synthesis of nucleic acid in Jensen rat sarcomas was inhibited following exposure to

x-rays according to Ahlström *et al.* (161). These investigators (162 to 166) studied nucleic acid synthesis with radioactive phosphorus. They found that x-rays inhibited nucleic acid synthesis in tumors and normal tissues by one-third to one-half for two hours following the radiation treatment. After this period, two-thirds of the inhibition was relieved.

A report has been made (167) on the distribution of nucleic acid within the cells of rat liver and hepatoma. By differential centrifugation, homogenates of the tissues were separated into three fractions, a nuclear fraction, a large granule fraction composed of mitochondria and secretory granules, and an unfractionated residue composed of microsomes and soluble protein complexes. The desoxyribonucleic acid was all recovered in the nuclear fraction of both tissues and more than twice as much was found in hepatoma as in liver. The increased content was considered to be due to an increase in the number of cells. The ribonucleic acid content of the two tissues was approximately the same, with the major portion occurring in the unfractionated residue. The content of ribonucleic acid per unit dry weight was higher in the tumor in both these fractions, but the lowered amount of cytoplasm in the hepatomas was responsible for the equivalent totals. A similar fractionation was reported (168) for Jensen sarcoma with essentially the same results.

Enzymes.—A considerable number of papers have appeared on the assay of enzymes in tumors and homologous tissues. Cathetic activity was reported higher in tumors than in normal tissues, but was correlated with the amount of necrosis in the tumors (169). In studies of the enzymatic deamination and dephosphorylation of nucleic acids in tissue extracts (170) it was found that desoxyribonucleic acid was attacked very slowly by tumor extracts; ribonucleic acid was attacked much more rapidly. Hepatoma was an exception and attacked both nucleic acids at equal rates and much more rapidly than did normal liver. In another report (171) the enzymatic activity of tissue extracts on ribonucleic acid was much higher in normal tissue than in tumor. Greenstein *et al.* (172) found that β -glycerophosphatase activity at neutral pH was lower in mouse tumors, with the exception of thymoma, than in normal tissues, with the range of values much smaller for the tumors. A low activity of glutaminase and asparaginase was observed for primary hepatomas (173). Pyruvic acid accelerated deamidation

without itself disappearing in extracts of normal tissues (174). This was explained on the basis of a condensation of pyruvic acid with the amino acid amide to form a dehydropeptide which could be split by dehydropeptidase to form ammonia and pyruvic acid. Extracts of rat hepatoma were stimulated very little by the pyruvate, presumably because of failure of the condensation reaction in tumor. This was supported by finding greater activity of dehydropeptidases in tumor than in normal tissues (175, 176). The possibility that dehydropeptides might be biological intermediates in protein metabolism was suggested (173). Fishman & Analyan (177, 178) found a very marked increase in β -glucuronidase activity of involved lymph nodes in breast carcinoma of humans and as high as a thirty-six hundredfold increase of activity in tumor compared to the surrounding tissues. The function of this enzyme in tumor metabolism is not yet clear.

Hirshfeld *et al.* (179), using potato tyrosinase as the test system, report an enzyme inhibitor occurring in the serum of cancer patients, but not in serum from normal individuals. This was not confirmed in a smaller series studied by Stadie *et al.* (180).

Lasnitzki (181), in support of the finding that continuous injection of sodium tannate strongly inhibited growth of mouse carcinoma transplants, found a marked inhibition of anaerobic glycolysis in Jensen sarcoma by low concentrations of tannin.

Several studies have been reported of enzyme assays on tissues during the carcinogenic process. Cohen (182) noted a reduction in the glyoxalase activity in the livers of rats fed *p*-dimethylaminoazobenzene; the hepatic tumors contained only 10 per cent of the activity of normal liver. Regenerating liver had normal activity. Succinoxidase activity of rat liver slices decreased when *p*-dimethylaminoazobenzene was fed and was even lower in the resulting tumors. This decrease in activity was prevented by diets that retard carcinogenesis (183). A similar decrease in enzyme was not observed when homogenates were used in place of liver slices or when 2-acetylaminofluorene was fed (184). These contradictory findings might be explained by the fact that homogenate assays were carried out without calcium and aluminum additions so that only a fraction of the potential activity was measured.

No change in oxidative metabolism or glycolysis was observed in the livers of mice fed *o*-aminoazotoluene, but a characteristic

decrease in oxidative metabolism and a high rate of glycolysis were observed in the resulting tumors (185).

Carruthers & Suntzeff (186) extended their studies of methylcholanthrene carcinogenesis in mouse epidermis to measurements of succinic dehydrogenase and cytochrome oxidase. Succinic dehydrogenase was very low in the normal epidermis, remained unchanged until the squamous cell carcinomas appeared, and in these was fourfold higher. The cytochrome oxidase rose soon after painting was begun, was twice the normal in the hyperplastic epidermis, fell somewhat on development of tumors, but was then still slightly above the normal. Although this is the reverse of earlier findings of Schneider & Potter (187) for certain other tumors, it is an excellent illustration of the observation that the range of enzyme activity is narrower among tumors than among normal tissues. Adenosinetriphosphatase activity (188) of tumors was within the range for the normal tissues assayed. Roberts & Carruthers (189) found a relatively low adenosinetriphosphatase activity in normal skin and a marked rise in its activity in the epidermal carcinoma. The findings of Schneider & Potter (187) and Carruthers & Suntzeff (186) are in agreement when the ratios of oxidative enzymes to adenosinetriphosphatase are compared.

Several enzymes appear to be completely lacking in tumors, though distributed generally throughout normal tissues. Losses such as the enzymes of urea synthesis in the transformation of liver cells to hepatoma are not surprising, since this is a specialized system not needed by the tumor. However, the absence of others cannot be explained on this basis at present and may provide useful leads toward definition of the carcinogenic change. Lan (190) found no choline oxidase in hepatoma 31, and 3 to 5 per cent as much D-amino acid oxidase as in liver; furthermore, D-amino acid oxidase, choline oxidase and uricase activities were completely absent from Walker 256 carcinosarcoma. No histidinase was found in tumors but the presence of a neoplasm of the liver resulted in a rise in the activity of this enzyme in the residual normal portion of this organ in mice; no such effect was observed in similarly involved human livers (191). A complete absence from tumors of cystine oxidase, exocystine desulfurase, cysteine dehydropeptidase (192, 193, 194) and of oxaloacetic acid oxidase (195) has been reported. Potter points out that Mitchell & Houlihan (196) have

found an alternate pathway in *Neurospora* open to oxaloacetic acid, in which it is converted to a nucleic acid component (uracil) via orotic acid. This assumes added importance when it is recalled that Plentl & Schoenheimer (197) found that isotopically labelled guanine, uracil and thymine were not utilized for nucleic acid synthesis when fed to rats. It may therefore be that tumor achieves added efficiency for pyrimidine synthesis by lacking this respiratory enzyme which catalyzes a high-energy-yielding reaction in differentiated tissues.

Schneider (167) has investigated the intracellular distribution of several enzymes (succinic dehydrogenase, cytochrome oxidase, adenosinetriphosphatase) in normal rat liver and hepatoma. Succinic dehydrogenase and cytochrome oxidase were found associated with the "large granule" fraction in both tissues and the activities were approximately the same per unit of protein, but the activity of these enzymes in tumor was much lower, due to less material of the large-granule type. The adenosinetriphosphatase activity was essentially the same in both tissues but the distribution was different; liver had most of the activity associated with the large granules, while hepatoma had most of it associated with the unfractionated residue.

The view has been expressed by a number of authors that tumor represents a distinct tissue entity, one tumor resembling other tumors rather than its homologous differentiated tissue. This is increasingly borne out as data accumulate for the vitamin content, the chemical makeup and the enzyme pattern of tumors.

Particularly with respect to enzyme studies, it appears that cancer research is reaching the point where comparisons between tumors and their tissues of origin are of secondary importance to a direct study of the enzyme pattern required by the tumors for their basic function, i.e., growth. However, as the two approaches are continued it will be possible to evaluate the significance of various enzyme systems to growth and to specialization, and greater emphasis can be placed upon the study of the enzyme pattern of malignant tissue. Thus our efforts toward the larger issues of diagnosis, prophylaxis and therapy will be concentrated on a smaller area.

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BIOCHEMISTRY OF THE NATURAL PIGMENTS

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In accordance with the request of the Editors, this review is exclusively devoted to the natural pyrrole pigments, such as hemes, porphyrins and bile pigments, which have not received adequate attention in this *Review* since the article of Holden (1) in 1945. Carotenoid and indolic biochromes of animals were thoroughly covered last year by Fox (2). The following reviews of other pigments have been recently published elsewhere: "Chemistry of the Pteridines" [Gates (3)], "Biochemistry of the Pterins" [Polonovski & Busnel (4)], "Pigments and Antibiotics of Fungi and Bacteria" [Sannié (5)] and "Algal Pigments and Their Significance" [Cook (6)].

The heme-containing enzymes, such as the cytochromes, catalase and peroxidase are treated elsewhere in this volume (p. 1-34).

The author wishes to pay tribute to the overwhelming contribution to pyrrole chemistry by Hans Fischer, whose tragic death in 1945 is a severe loss.

Reviewing recent work on pyrrole pigments, the author has the impression that greater clarity and new information might be gained by applying more precise methods of separation and characterization. Many authors are satisfied with spectroscopic identification of pigments, where a more detailed investigation would show the complexity of the material studied. The mixed chromatogram is more and more widely used for comparison of two pigments, while a still greater precision can be attained by using three tubes simultaneously (two for the pure pigments, the third for the mixture of both). In this way the chromatographic purity of each pigment is more easily assessed, particularly where two pigments are adsorbed at slightly different heights, or with slightly different colors, but form a homogenous zone on one column (7). The recent paper of Strain (8) on reversal of the sequence of zones by different solvent mixtures suggests a new possibility in testing the identity of two pigments.

There is still much work to be done and important progress

¹ The author wishes to thank Dr. J. W. Legge for helpful criticism of this manuscript.

may be attained by elaboration of chromatographic methods for separating heme chromoproteins. While chromatography of porphyrins and bile pigments has been employed with success [Watson *et al.* (9, 10), Gray & Holt (11), Lederer & Tixier (12), Tixier (13)], separation of heme pigments is still very difficult [Holden (14), Liébecq (15)]. Paper chromatography furnishes a new technique for separating and identifying small quantities of biochromes.

Whereas prior to 1940, the principal interest centered on elucidation of the breakdown of hemoglobin to bile pigments, recent progress concerns the biosynthesis of hemoglobin. Researches on hemoglobin of invertebrates, leguminous root nodules, and microorganisms bear out the universal capacity of living cells to synthesize heme chromoproteins.

Holden's criticism (1) of the unsatisfactory state of nomenclature in the field under review is still valid; it is hoped that a general agreement will soon be reached in this matter.

HEME PIGMENTS

HEMOGLOBIN

A special report of the Committee on hemoglobin surveys of the Medical Research Council (16) contains results of measurement of the hemoglobin level in about 13,000 adults and 3,000 children, in England, 1943. Whereas the general situation was reasonably good, low levels were frequently found in young children, pregnant women, and persons at the lower economic levels. The report contains a detailed review of physiological factors influencing hemoglobin levels and a discussion (by Macfarlane) of the error of hemoglobin estimation by the Haldane-Gowers method.

Barcroft, in a lecture on "The Usefulness of Hemoglobin" (17), considers the role of hemoglobin as a carrier and in storage of oxygen for animals and plants; special stress is laid on the significance of the oxygen dissociation curve and the fact that hemoglobins with different affinities for oxygen serve the requirements of creatures living in widely different environments.

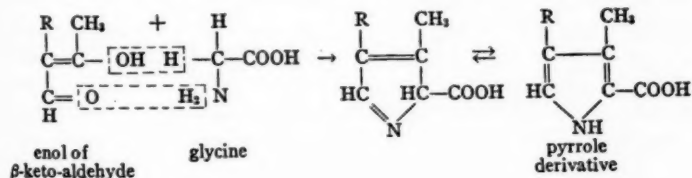
Biosynthesis of hemoglobin.—The most interesting results in this field are due to the isotope work of Bloch, Shemin & Rittenberg (18, 19, 20). By feeding deuterioacetate to rats and by isolating D-containing protoporphyrin from the hemoglobin of these animals, Bloch & Rittenberg (18) showed in 1945 that some of the carbon atoms of the side chains of heme are derived from acetic

acid, for none of the carbon atoms of the pyrrole ring is bonded with hydrogen. Subsequently, Shemin & Rittenberg (19) fed glycine containing N^{15} to humans; the protoporphyrin isolated from their blood contained a high percentage of N^{15} ; the authors conclude that protoporphyrin is synthesized from glycine and either acetic acid, or some compound closely related to it.

Recently, Shemin & Rittenberg (20) have reported more extensive experiments with N^{15} -labeled glycine, glutamic acid, proline, leucine, and ammonia. Proline and glutamic acid were selected since it has often been suggested [though not invariably accepted (21)] that proline and the anhydride of glutamic acid, pyrrolidone-carboxylic acid, may be precursors of the porphyrins. Leucine was chosen as a representative α -amino acid whose intact carbon chain is unlikely to be used for pyrrole synthesis; ammonia was chosen in order to test the nonspecific utilization of nitrogen liberated by deamination of amino acids. The results clearly demonstrated that glycine is by far the most effective source of nitrogen for the synthesis of heme. The isotope concentrations found in the porphyrin after feeding of ammonia, glutamic acid, proline and leucine are not significantly different from each other but are from 8 to 20 per cent of that found after feeding glycine. This indicates that the nitrogen of glycine is directly employed for the synthesis of protoporphyrin, while the nitrogen of the other compounds is used indirectly, presumably by way of glycine. While it is so far only proven that the nitrogen of glycine is utilized for the synthesis of porphyrin the authors do not seem to doubt that this conversion involves both carbon atoms of glycine as well.

As Fischer & Fink (22) had shown that formylacetone and glycine condense readily to yield a product which gives reactions of pyrroles, Shemin & Rittenberg (19) conclude that the biosynthesis of protoporphyrin probably involves the condensation of glycine with a β -keto-aldehyde formed, in part at least, from acetic acid.

The reaction may occur as shown in the accompanying diagram; according to this formulation, one α -carbon atom of the pyrrole



rings and the carbon atoms of the methine bridges are derived from glycine.

Shemin & Rittenberg (23) then followed the fate of N^{15} -containing heme in man over a long period. After administration of N^{15} -containing glycine, the N^{15} values of hemoglobin rose rapidly to a high level, remained practically constant for many weeks, and then fell quite sharply to a very low level. Mathematical analysis indicated the remarkable fact that hemoglobin is not involved in the dynamic metabolic state characteristic of most other proteins. The red cell is supplied with its store of hemoglobin which remains intact during its life time. The heme N^{15} liberated by hemoglobin breakdown is not reutilized for hemoglobin formation. By plotting the N^{15} concentration of heme against time, the authors have determined the average life span of the red blood cell. This was found to be about 127 days, in good agreement with some previous estimates and recent analogous experiments of Hevesy (24).

Orten & Orten (25) studied the comparative value of certain dietary proteins for hemopoiesis in the rat; they observed a general parallelism between the hemopoietic value of the protein and its ability to support somatic growth and noted the very efficient utilization of limited amounts of ingested protein for the regeneration of hemoglobin. Orten & Keller (26) have studied the influence of dietary protein on the protoporphyrin excretion of the rat. Assuming that protoporphyrin excretion is a measure of porphyrin synthesis in the organism, they conclude that dietary protein serves as a precursor of the porphyrin nucleus in the rat [thereby confirming the more precise results of Shemin & Rittenberg (19)] and that porphyrin formation, like hemoglobin formation, has a high "priority rating" for protein available in the organism.

Kornberg (27) and Benditt *et al.* (28) found that a mixture of the ten essential amino acids can largely replace casein for erythrocyte production of the rat. Arginine does not seem to be indispensable (28). An analogous study by Robschey-Robbins *et al.* (29) on the dog shows that whereas tryptophane (and to a lesser extent, phenylalanine and threonine) favors output of plasma protein, histidine, arginine, and lysine, on the contrary, favor hemoglobin synthesis. Whipple *et al.* (30, 31) report detailed studies of the effect of various proteins and amino acid mixtures on hemoglobin and plasma protein synthesis in anemic and hypoproteinemic dogs.

Yeshoda & Damodaran note (32) that tryptophane accelerates

markedly the regeneration of hemoglobin in rats suffering from anemia induced by phenylhydrazine.

The iron necessary for hemoglobin synthesis is furnished by ferritin, the brown iron-containing protein present in spleen, bone marrow and liver. The iron in ferritin is stored in the form of colloidal micelles of very insoluble basic iron phosphate attached to a relatively large nondiffusible protein molecule, apoferritin [Granick (33) Michaelis (34)]. When iron is required for heme synthesis, it is made rapidly available, probably by reduction of ferritin iron to the ferrous form. Granick (33) supposes that at the same time demands for globin synthesis are met in part by breaking down the apoferritin molecule itself.

The factors governing the very complex biological systems affecting hemopoiesis have been reviewed by Pffner & Hogan (35).

Physicochemical studies.—Rubinshtein & Ravikovich (36, 37), confirm the conclusions of Keilin & Hartree (38) and show that the missing of the γ absorption band of hemoglobin in the erythrocytes (at 425 $m\mu$) is a purely optical phenomenon due to light scattering, as this band may be detected by a selenium photocell. Thus, the hemoglobin of red cells is in the same chemical and physical state as in aqueous solution.

Corwin & Erdman (39) have prepared pure mesoheme IX dimethyl ester, the pyridine hemochromogen of which is passive to oxygen in absence of water, or in the presence of not more than the stoichiometric quantity. In biological systems, however, the oxidation is apparently independent of the amount of water. The authors suggest that

a protein could reduce the amount of water available to the iron atom if it were a large concave surface capable of partially engulfing the heme molecule with suitable coordinating groups spatially arranged so as to be immediately available geometrically for attachment to the two free coordinating valencies of the iron.

A single linkage would not permit advancing a geometric argument of the type proposed. For certain ferriheme compounds, as for instance the pyridine complex of hematin, Erdman & Corwin (40) discuss the existence of seven covalent bonds to the iron atom. They have also considered the nature of the N-H bond in porphyrins; hydrogen bridging does not seem to occur to an appreciable extent (41).

Pauli & Schwarzscher (42) describe the preparation of pure oxyhemoglobin which in a 4 to 5 per cent solution has the same

conductivity as distilled water and a pH of 7.048 to 6.92. They studied in detail the pH dependence of viscosity and other electrochemical features.

Drabkin has published a detailed crystallographic and optical study of hemoglobin of man in comparison with other species (43).

McCarthy & Popják (44) have measured the osmotic pressure of fetal sheep hemoglobin; their results suggest a dissociation of fetal hemoglobin into smaller molecules on dilution. In more concentrated solutions the molecular weights of maternal and fetal hemoglobin seem identical. That fetal and adult bovine hemoglobins are two different proteins is again illustrated by the difference in their methionine and isoleucine content [Brand & Grantham (45)].

Contrary to Darling & Roughton (46), Boeri & Vescia (47) do not accept the existence of intermediate compounds between hemoglobin (wholly ferrous) and methemoglobin (wholly ferric).

Biochemical studies.—Gibson & Harrison (48) have described a stable artificial standard solution which matches the absorption spectrum of blood in 0.1*N* NaOH over a considerable range. They have applied their method to the study of the relationship between oxygen capacity and iron content of blood in men and women (49). The ratio between oxygen capacity and iron content is the same in the blood of both sexes. The specific oxygen capacity found was 393 cc. O₂ per gm. of iron compared with the stoichiometric value of 401 cc.

Van Slyke *et al.* (50) have described improved techniques for determining blood carbon monoxide and total active and inactive hemoglobin by the carbon monoxide capacity procedure. The term "active Hb" is used to indicate hemoglobin capable of combining reversibly with oxygen or carbon monoxide; "inactive Hb" to indicate hemoglobin derivatives that do not combine reversibly with oxygen or carbon monoxide but can be converted into active Hb by reducing agents that convert ferrihemoglobin into ferrohemoglobin. "Methemoglobin" is used to indicate inactive Hb which in addition to the ability to be converted into active Hb by reducing agents also shows, on treatment with cyanide, the changes in optical density characteristic of methemoglobin.

The mean methemoglobin found by Van Slyke *et al.* was only 0.4 per cent of total Hb; in about half the blood samples the percentage was so low (0.0 to 0.3) that the presence of methemoglobin

was uncertain. This confirms the findings of Paul & Kemp (51) [see also Ramsay (52)]. The mean inactive Hb of freshly drawn blood was 1.3 ± 0.35 per cent of total Hb. This means that there is about 1 per cent of ferrihemoglobin which is not methemoglobin. This pigment is rapidly transformed into hemoglobin when the blood is permitted to stand for two hours [see also Kallner (53)]. These values are in agreement with those of Drabkin & Schmidt (54) concerning the degree of saturation of hemoglobin in arterial blood (average 98.5 per cent).

Van Slyke *et al.* (50) point out that a survey of pathological blood samples with simultaneous photometric and gasometric hemoglobin values and iron determinations is desirable to ascertain what pathological conditions are likely to produce anomalous hemoglobin derivatives, such as in the case of idiopathic cyanosis reported by Roughton, Darling & Root (55).

Brown *et al.* (56, 57) have studied the normal variations in human blood hemoglobin concentration and the effects of epinephrine; increases up to 11 per cent have been observed after injection of 1 mg. epinephrine (57).

Foster (58) has reported quantitative determinations of aspartic and glutamic acids, leucine, glycine, lysine, arginine, phenylalanine, and tyrosine by the isotope dilution method on acid hydrolysates of horse hemoglobin. Human, horse and adult bovine hemoglobins contain no isoleucine, contrary to dog and fetal bovine hemoglobins [Brand & Grantham (45)]. Roche & Michel (59) have studied the threonine content of hemoglobins of different species and found variations from 2.6 to 6.4 per cent. The elegant method of Sanger (60) for determining the free endgroups of proteins by reaction with 2,4-dinitro-fluorobenzene has given some valuable information about the species differences in the globins of mammals. Thus, human hemoglobin has five valine endgroups, horse and donkey hemoglobin six, whereas sheep, cow and goat hemoglobins have two valine and two methionine endgroups (61). These studies may shed new light on the problem of the specificity and immunological behavior of hemoglobins.

Papers by Davis & Ross (62), Liébecq (63), and Nizet (64) concern the action of mustard gas, chloropicrin, and phenylhydrazine on hemoglobin.

Keilin & Hartree (65) have described a new method for the study of the permeability and lysis of erythrocytes based upon the

rapid reactions of hemoglobin and methemoglobin with a number of substances, resulting in the formation of spectroscopically well-defined and easily recognizable compounds.

Methemoglobin.—Albaum *et al.* (66) have studied the competition of methemoglobin with cytochrome oxidase for potassium cyanide. Methemoglobin can reverse *in vitro* the cyanide inhibition of cytochrome oxidase; this reaction explains the beneficial effects of methemoglobinemia induced *in vivo* to counteract the effects of cyanide. Ascorbic acid reduces *in vitro* the methemoglobin of the red cells of patients with familial idiopathic methemoglobinemia to normal hemoglobin; the treatment of this illness with ascorbic acid [Barcroft *et al.* (67), Carnrick *et al.* (68)] or with methylene blue [Bodansky *et al.* (69)] seems promising.

Disintegration of hemoglobin to bile pigments.—No important new facts have been published in recent years concerning this biochemical problem. The reviews of Polonovski & Gajdos (70) and of Liébecq (71) cover this question in detail [see also Watson (72)].

Contrary to recent opinions that hematin can not be transformed into bile pigments *in vivo*, Watson and co-workers (73) (in man) and Polonovski and co-workers (74) (in dog) have shown that hematin can, at least partially, be converted into bilirubin. This is of interest because of the presence of hematin in the circulating blood in a variety of pathological states, such as gas bacillus sepsis, pernicious anemia and black water fever (73).

It would be important to verify the claims of Kesztyüs & Kiese (75) of having isolated enzymes from liver pulp which act specifically on the formation of bile pigments from hemoglobin. At pH 7.5, hemoglobin and verdoglobin are transformed into bile pigments; the reaction verdoglobin→bile pigments is much more rapid than the formation of bile pigments from hemoglobin. The two stages hemoglobin→verdoglobin→bile pigments can be separated at pH 5.2, where liver extracts produce bile pigments from verdoglobin, but leave hemoglobin unchanged. The formation of verdoglobin from hemoglobin can be observed at pH 7.4. The activity of the liver extract is lost by dialysis, but can be restored by addition of boiled liver juice.

The spectral characteristics of the verdoglobin of Kesztyüs & Kiese (wavelength max. 620 μ ; carbon monoxide myoglobin max. 615 to 620 μ) seem to suggest the presence of sulfhemoglobin.

In this connection the observation of Barron *et al.* (76) is of

interest, that in the presence of oxygen, dithiols destroy iron porphyrin compounds by opening the porphyrin ring.

Holden (77) has continued his study of "cruoralbum," a green pigment formed by alternate reduction and reoxidation of oxy-hemoglobin in the presence of hydrogen cyanide. The prosthetic group "cruoratin" appears to retain an intact porphyrin ring but is very unstable, being readily destroyed by oxidants or acids. No evidence for the production of bile pigments during its destruction could be found. In a subsequent paper, Holden (14) reports chromatographic experiments for preparation of pure cruoratin.

Liébecq, in a series of papers (78 to 83) on the pseudohemoglobin of Barkan & Schales (84) has stressed the complex character of this pigment, which is said to contain the following substances: cyancathemoglobin, cyanmethemoglobin, pseudocyancathemoglobin, pseudocyanmethemoglobin and some colorless proteins. Pseudohemoglobin is finally shown to be identical with the choleglobin of Lemberg, Legge & Lockwood (85).

Granick (33) has stressed the fact that the iron released from hemoglobin breakdown, unlike the porphyrin moiety, is carefully conserved by the body by combination with apoferritin to form ferritin.

Hemoglobin of invertebrates.—Keilin & Wang (86) report an interesting study of the crystalline hemoglobin of the tracheal cells of the larvae of the botfly (*Gastrophilus intestinalis*) which live in the stomach of the horse. This hemoglobin, which enables the larvae to live in a medium having only an intermittent contact with air, crystallizes readily, contains 3.92 per cent hemin, corresponding to an equivalent weight of 17,300 and has a molecular weight of about 34,000 [Adair *et al.* (87)], i.e., about half the molecular weight of the hemoglobin of vertebrates. The *Gastrophilus* hemoglobin therefore contains only two heme nuclei. It differs also from horse hemoglobin by the position of absorption bands, the relative affinities for oxygen and carbon monoxide, the shape of the dissociation curve and the ease of autoxidation. Since both hemoglobins have the same prosthetic group, they differ in structure and composition of their proteins; the larvae therefore synthesize the globin part of their hemoglobin. It is not known whether they also synthesize the prosthetic group, or use that of the horse. In this respect, Lwoff & Nicolle (88) report that artificially fed larvae of the assassin bug *Triatoma infestans* can not synthesize

hematin which acts as a growth factor for these insects.

The paper of Keilin & Wang (86) contains a detailed description of an apparatus for the spectroscopic study of the oxygen dissociation of hemoglobins.

The hemoglobins of *Gastrophilus* and the leguminous root nodules (*vide infra*) and the myoglobins of vertebrates are all localized within the fixed noncirculating cells, and constitute a group of pigments characterized by a high affinity for oxygen, low affinity for carbon monoxide, by their ease of oxidation and low molecular weight; this group "reduces somewhat the gap separating the two groups of hematin protein compounds, the oxygen carriers and certain oxidizing catalysts" [Keilin & Wang (86)].

Davenport (89) has studied hemoglobins with very high affinity for oxygen, found in the body walls and the perienteric fluid of *Ascaris lumbricoides*. Pérez & Bloch-Raphaël (90) have found a hemoglobin and bile pigments in the Rhizocephale *Septosaccus Cuénoti*, a parasite of the arthropode *Diogenes pugilator*, which does not contain any hemoglobin at all. Fox (91) has recently studied the role of hemoglobin in *Daphnia*. An increase of blood pigment was observed in response to oxygen deficiency, but when the hemoglobin was inactivated by carbon monoxide, the animals survived as long as untreated ones. In the eggs hemoglobin seems necessary because of lack of oxygen in the brood pouch. Fox finally considers the possibility

that hemoglobin still appears in the blood of *Daphnia* as a functionless by-product of metabolism, as it must once have done in the early history of animals, before natural selection seized upon its potentially valuable property of reversible oxygenation.

Hemoglobin of leguminous root nodules.—Kubo (92) first observed the great spectral similarity of the red pigment of the leguminous root nodules with hemoglobin and obtained from it crystals of hemin. Keilin & Wang (93) have since shown clearly that, like hemoglobin, this pigment forms a loose and perfectly reversible compound with molecular oxygen. Its iron, like that of oxyhemoglobin, is in a divalent state, as shown by its reaction with carbon monoxide and potassium ferricyanide. The relative affinity of this pigment for oxygen and carbon monoxide, expressed as the equilibrium constant $k = \frac{[\text{HbCO}] [\text{pO}_2]}{[\text{HbO}_2] [\text{pCO}]}$, is about 37. Corresponding

values for hemoglobin of vertebrates and of *Gastrophilus* are 120 to 550, and 0.67, respectively. The value for mammalian myoglobin is 28 to 51; for phenol oxidase, 0.25 to 1; and for cytochrome oxidase, 0.1 (86).

Simultaneously, Virtanen and his school (94 to 97), working on symbiotic nitrogen fixation, confirmed the basic observations of Keilin & Wang. Virtanen & Laine (96) report, in addition to the red pigment, hemoglobin, the presence of brown and green pigments in leguminous root nodules. The brown pigment is said to be methemoglobin with trivalent iron.

If the plants are held in the dark, or at the end of the flowering, the nodules turn green and fix no more nitrogen. The green pigment can be isolated from the nodules in the same way as the red and brown pigments; it is a chromoprotein with a positive Gmelin reaction and it contains divalent iron (about 0.29 per cent iron) [Virtanen *et al.* (97)]. The green pigment seems to belong to the precursors of the bile pigments and resembles the choleglobin of Lemberg *et al.* (85). In the first papers, Virtanen ascribed to oxaloacetic acid an important role in nitrogen fixation by leguminous root nodules, as it was said to reduce methemoglobin to hemoglobin.

Keilin & Smith (98) examining sliced root nodules with the microspectroscope were unable to confirm the presence of methemoglobin in the fresh root nodules. The appearance of methemoglobin in Virtanen & Laine's experiments is explained by their use of nodule extracts in which hemoglobin undergoes oxidation by quinones formed during extraction. Keilin & Smith state also that oxaloacetic acid reduces neither methemoglobin nor any other hematin compound. They conclude:

within the root nodules of leguminous plants hemoglobin displays its usual property of oxygenation ($\text{Hb} + \text{O}_2 = \text{HbO}_2$). . . . Moreover it seems unlikely that oxidation reactions in nodules are catalysed by a compound such as hemoglobin which has the remarkable property of oxygenation and is therefore an excellent oxygen carrier, but a very inefficient catalyst.

Little & Burris (99) have confirmed the identity of the position of the absorption bands of legume hemoglobin and several of its derivatives with those of hog hemoglobin. Hemoglobin stimulated the rate of oxygen uptake by the rhizobia taken directly from the soybean nodules and by washed suspensions of pure cultures of

Rhizobium trifolii. The nodule pigment causes a similar, but less marked, stimulation. Little & Burris think that oxygen is a limiting factor for the respiration of the bacteria packed in the active tissue of root nodules and see the most probable role of legume hemoglobin in aiding in the rapid release of oxygen within the nodule.

Virtanen *et al.* (97) have recently described the preparation of pure legume hemoglobin containing 0.34 per cent iron and having a molecular weight of 34,000, half that of hemoglobin of blood; the former therefore contains two heme groups per molecule.

Vennesland, Evans & Francis (100) have made an observation which might have a bearing on the role of the legume pigments; crystalline metmyoglobin, in presence of oxygen and manganese, causes the oxidative decarboxylation of oxaloacetic acid to malonic acid and carbon dioxide; hemoglobin and cytochrome-*c* were inactive. During the oxidation the metmyoglobin is converted first to a green pigment and then to a light brown pigment which shows no sharp absorption bands in the visible spectrum and is inactive in the test system.

The following lines of Keilin & Smith (98) best sum up the question:

... the fact that this hemoglobin is present in root nodules of every leguminous plant, that this pigment is formed only in nodules produced by an "efficient" strain of *Rhizobium*, that the pigment is localized only within the large cells containing the symbiotic organisms and that nitrogen fixation by nodules is strongly inhibited by small concentration of carbon monoxide, leave little doubt that the activity of hemoglobin is linked with the process of symbiotic nitrogen fixation.

MYOGLOBIN

Theorell & de Duve (101) have isolated crystalline human myoglobin from heart muscle and from the urine of patients with mild paralytic myoglobinuria; both preparations were shown to be identical. Myoglobin can be separated from hemoglobin by salting out; its iron content is 0.34 per cent, like that of horse myoglobin and human hemoglobin. The authors confirm the relation of myoglobinuria with carbohydrate metabolism by mentioning that one of their patients, put on a carbohydrate free diet, developed an extremely severe attack of myoglobinuria. No hemoglobin was found in these urines.

Crandall & Drabkin (102) report that a rat of 250 gm. body weight contains 3190 mg. hemoglobin, 101 mg. myoglobin and

14.4 mg. cytochrome-*c*; the relative proportions of these three pigments vary considerably for different species.

Bücher & Kaspers (103) have studied the photochemical scission of carbon monoxide myoglobin by monochromatic ultraviolet light. It was found that not only the radiation absorbed by the hemin constituent, but also that absorbed by the globin is effective in splitting carbon monoxide from carbon monoxide myoglobin.

Sanger & Porter (61) have found that horse myoglobin (mol. wt. 17,000) has one terminal glycine residue per molecule, in sharp contrast to horse hemoglobin (mol. wt. 64,000) which has six terminal valine residues per molecule.

In view of the pronounced differences in the globin part of myo- and hemoglobin, the claim of Wajda (104) concerning the origin of erythrocytes from striated muscle seems rather astonishing, as it would imply a transformation of myoglobin to hemoglobin during erythropoiesis.

PORPHYRINS

Protoporphyrin.—Chu (105) and Grinstein (106) have devised improved methods of preparing pure protoporphyrin IX dimethyl-ester from hemoglobin. Chu (107) has described a qualitative test to distinguish between protoporphyrin IX or its esters and porphyrins containing no vinyl groups; dissolved in chloroform containing concentrated hydrochloric acid, protoporphyrin gives a green, mesoporphyrin a purple color.

Watson (72) has made a detailed clinical study on the presence of free protoporphyrin in the erythrocytes; one or more of at least three factors appear to be concerned in determining the amount of protoporphyrin in the erythrocytes: (a) increased reticulocyte percentage (reticulocytes being rich in protoporphyrin), or normoblastic hyperplasia, in the bone marrow (b) iron deficiency, or factors interfering with the utilization of iron in the synthesis of hemoglobin, as for example lead; (c) the formation of protoporphyrin from hemoglobin in the erythrocytes. The normal range of concentration of the erythrocyte protoporphyrin is from 15 to 40 μg . per 100 ml. of erythrocytes, usually below 30 μg . Values as high as 600 μg . per cent have been found in iron deficiency anemia. Watson stresses the clinical value of the erythrocyte protoporphyrin determination for the diagnosis of different anemias.

Orten & Keller (26) have reviewed some aspects of protopor-

phyrin metabolism in the rat and Zeligman (108) has found no evidence that the rat is able to convert protoporphyrin to coproporphyrin.

Towbin *et al.* (109) have identified the porphyrin of the Harder gland of the rat as protoporphyrin; it is this pigment and not hemoglobin that appears in choline-induced chromodacryorrhea. Raoul & Marnay (110, 111, 112) have studied a generalized cutaneous excretion of protoporphyrin in rats fed a diet deficient in pantothenic acid and pyridoxine and described by some authors as "rustiness." The "blood caked whiskers" observed by others are also due to excreted protoporphyrin. The cutaneous protoporphyrin excretion is accompanied by an increased urinary excretion of coproporphyrin and the appearance of free protoporphyrin in the liver.

Lederer & Tixier (12) have shown that the "ambraporphyrin" of amber gris (113) is not a pure substance, but a mixture of protoporphyrin IX and mesoporphyrin IX which can be separated chromatographically. Mesoporphyrin exists also in the intestine of humans and is probably formed by bacterial reduction of protoporphyrin.

Coproporphyrins.—Jope & O'Brien (114) have found the spectral absorption and fluorescence of coproporphyrin isomers I and III to be identical and have studied criteria of purity of the tetramethyl-esters. Schwartz *et al.* (115, 116) describe the quantitative differentiation of small amounts of the coproporphyrin isomers I and III based on the quenching of the fluorescence of coproporphyrin I in aqueous acetone.

Watson *et al.* (117) have found a marked coproporphyrinuria (type III) in acute poliomyelitis; (100 to 500 μ g. coproporphyrin per twenty-four hours with 50 to 90 per cent type III isomer, instead of the normal values of 20 to 100 μ g. with 8 to 35 per cent of type III isomer.)

Rimington & Leitner (118) have studied the urinary excretion of coproporphyrin in nonalcoholic pellagra and conclude that excess porphyrinuria is not an essential feature of pellagra. Apparently a combination of circumstances (e.g., alcoholism plus certain nutritional deficiencies) is needed to produce liver damage with consequent porphyrinuria.

Dent & Rimington (119) have recently observed a marked porphyrinuria in rats fed oxidized casein; methionine-sulphone (pres-

ent in the oxidized casein) is not toxic, but a peptide containing methionine-sulphone may be the porphyrigenic agent. The exact nature of the porphyrin was not determined.

Watson & Larson (120) have recently published a very detailed and instructive review "The Urinary Coproporphyrins in Health and Disease." Coproporphyrin excretion is discussed in relation to liver diseases, jaundice, blood diseases, hemorrhage, fever and infectious diseases, porphyria, effects of chemicals and heavy metals and skin diseases. Cases of excessive coproporphyrinuria without known cause, in which no uroporphyrin is found are classified as "idiopathic coproporphyrinuria." Daily urinary coproporphyrin excretions were observed up to 6 mg., practically all of which was the type III isomer.

Nothing definite is known concerning the formation of the coproporphyrins (by carboxylation of protoporphyrin or decarboxylation of uroporphyrin, or independently from both of these porphyrins). The conclusion of Watson & Larson (120) is: "Thus the exact site and mode of formation of the coproporphyrins and their physiological role remain in doubt."

A very interesting study of Klüber (121, 122) reports the presence of coproporphyrin in the white matter of the central nervous system; no porphyrin was detected in the pineal gland, hypophysis, chorioid plexus, cerebrospinal fluid, aqueous, and vitreous humor, or meninges of the brain and spinal cord; the only other tissue containing coproporphyrin was the lining of the gizzard of chick and pigeon. The presence of such a remarkably photodynamic substance as coproporphyrin in the optical nerve seems particularly significant. Klüber has observed a progressive "porphyrinization" of the central nervous system during postnatal development. No porphyrin is present at birth. Recalling the favorable results obtained with hematoporphyrin in certain psychoses, Klüber discusses the possibility that certain neurological and psychiatric disorders may be due to or associated with a "cerebral porphyria" or a disturbance of the metabolism of certain pyrrole compounds in the nervous system.

Kench & Wilkinson (123) have studied the coproporphyrin metabolism of yeast. They could detect no coproporphyrin as the result of breakdown of added catalase or cytochrome-*c*. Coproporphyrin seems to be an intermediate of metabolism, perhaps associated with high rates of cellular division.

Gray & Holt (124) have isolated pure coproporphyrin III from the culture medium of the diphtheria bacillus, where it is accompanied by small amounts of protoporphyrin (11). Pappenheimer (125) has studied the effect of iron on toxin and porphyrin production by the same bacillus. For every four atoms of iron added to the culture medium, four molecules of porphyrin and one toxin molecule disappear from the culture filtrate; the added iron is recovered quantitatively from the bacterial cells. The toxin is supposed to be the protein moiety of one of the iron-containing respiratory enzyme.

Watson & Larson (120) report the production of coproporphyrin III by certain strains of intestinal bacteria; there was no appreciable absorption or urinary excretion of the bacterial porphyrins. Klüver (122) states that copro- and protoporphyrins are present in several strains of penicillium and even in commercial preparations of penicillin.

Uroporphyrins.—Grinstein *et al.* (9) have used Tswett columns of calcium carbonate to obtain pure uroporphyrin I octamethyl ester m.p. 284°. Waldenström's uroporphyrin m.p. 258–260°, obtained from porphyria urine (126), was shown to be a mixture of the uroporphyrin I ester m.p. 284° and a new porphyrin ester m.p. 208° (probably a heptamethylester) with absorption spectra slightly different from the uroporphyrin spectra. Decarboxylation of the latter produced, in part, coproporphyrin III, but there was no indication of the occurrence in porphyria material of a uroporphyrin type III. Watson *et al.* (10) have studied porphyrins obtained according to Waldenström's method, melting variously from 258–272°. These could not be separated into two porphyrins on the calcium carbonate column; however, evidence is presented to show that this is a mixture of a large amount of Type I and a small amount of Type III isomers (the first being represented by uro- and coproporphyrins I, the second only by coproporphyrin III). Again there was no evidence for the existence of uroporphyrin III. Prunty (127) has reached similar conclusions. The following table of Watson *et al.* (10) (Table I) represents the characteristic differences observed in the porphyrins of the urine of the congenital and intermittent acute types of porphyria. It is remarkable that, principally in the intermittent acute type, nearly all the porphyrin is present as zinc complex.

Watson (128) has reviewed the more medical aspects of por-

phyria and Waldenström & Vahlquist (129) have studied the excretion of porphobilinogen in patients with acute porphyria. Prunty (130) has prepared purified solutions of porphobilinogen from the urine and liver of a case of acute porphyria and confirmed the formation of uroporphyrin from porphobilinogen heated in acid solution. There was no evidence for formation of porphyrin from porphobilinogen injected into the rabbit.

TABLE I
CHARACTERISTIC DIFFERENCES IN URINES OF CONGENITAL AND
INTERMITTENT ACUTE TYPES OF PORPHYRIA*

Congenital	Intermittent Acute
Majority or all of porphyrin in free state, not combined with zinc	All or most of porphyrin present as zinc complex
Relatively large amounts of uro- and coproporphyrin I	Small amounts of porphyrin consisting of a mixture of 260° porphyrin with coproporphyrin, either Type I or III, or a mixture
Porphobilinogen absent	Much porphobilinogen and porphobilin
Urine purer red in color	Urine exhibits browner tint

* From Watson *et al.* (10).

Klüver (122) states that in numerous mammals the auditory ossicles and the bony labyrinth contain a uroporphyrin.

Tixier (13, 131) has isolated crystalline uroporphyrin I octamethylester from the shells of the molluscs *Pteria macroptera* and *Clanculus pharaonis*; again there was no indication of the presence of uroporphyrin III.

Miscellaneous.—The claim of Figge (132) concerning a relationship between the porphyrin content of the Harderian glands of mice and susceptibility to mammary cancer has not been confirmed by Bittner & Watson (133); there seems to be no simple correlation between "inherited susceptibility" to mammary and lung cancer and the porphyrins.

Klüver (122) has observed a striking affinity of the hypophysis for injected hematoporphyrin; this might explain the estrogenic effect observed after injection of this porphyrin.

Granick & Gilder (134) studying the porphyrin requirements of *Haemophilus influenzae* have reached very interesting conclusions concerning the biochemical role of the side chains of the porphyrins. Only protoporphyrin, containing vinyl side chains, supports growth of *Haemophilus influenzae*, contrary to meso- and hematoporphyrin, which are lacking these vinyl groups. As the corresponding iron porphyrins are all active in restoring growth, Granick & Gilder conclude that the vinyl groups of protoporphyrin are necessary for the biological insertion of iron into the porphyrin molecule. The carboxyl groups of protoporphyrin, on the other hand, seem to be necessary for the attachment of the porphyrin to the specific apoproteins, since the methyl ester of protoporphyrin or of the iron porphyrins are all inactive. The methyl side chains are supposed to be necessary to "stabilize the rings in order to prevent any undesirable side reactions from occurring within the cells."

Porphyrins not containing vinyl groups may act as antagonists of protoporphyrin; the inhibition is competitive in nature. Granick & Gilder suggest that natural porphyrins, such as the coproporphyrins, could act as regulators of the rate of oxygen consumption by the cell.

Curd & Rose (135) suggest a possible mode of action of the antimalarial drug paludrine. Due to its structural resemblance with the porphyrins, it might interfere with the porphyrin metabolism or with a porphyrin-enzyme system of the parasite.

BILE PIGMENTS

Bilirubin.—With (136) has measured the spectral absorption curves of aqueous bilirubin solutions with and without addition of serum. In presence of serum the absorption curve is shifted to longer wavelengths. This is due to the combining of bilirubin with serum albumin, one molecule of albumin is thought to combine with twenty-five to fifty molecules of bilirubin, but Cohn *et al.* (137) have stated recently that one molecule of serum albumin combines with three moles of bilirubin. The stability of bilirubin is greatly enhanced by the presence of serum albumin; amino acids have no such effect [Barac & Roseman (138)].

Much discussion has been engaged in the past few years concerning the two forms, direct and indirect, of the van den Bergh diazo reaction with serum bilirubin. For Watson (72) the delayed

or indirect van den Bergh reaction (characteristic of retention jaundice) is due to bilirubinglobin, which is not excreted in the urine; the prompt or direct reacting van den Bergh reaction (characteristic of regurgitation jaundice), on the contrary, is due to sodium bilirubinate (loosely attached to serum protein) which is readily excreted in the urine. Lemberg (139) has not been able to confirm the findings of Fiessinger *et al.* (140) that bilirubinglobin behaves as indirect, and bilirubinalbumin as direct bilirubin.

Gray & Whidborne (141, 142) doubt the usefulness of the measurements of directly and indirectly reacting bilirubin of Ducci & Watson (143) and conclude that the rapid formation of "azobilirubin," on the addition of diazo reagent, to sera from patients with regurgitation jaundice is due to the presence of catalysts in the sera and not the existence, in regurgitation jaundice, of a form of bilirubin different from that in serum of patients with retention jaundice.

Gardikas *et al.* (144), on the contrary think that indirect bilirubin may be an intermediate precursor of bilirubin in hemoglobin breakdown, still attached to globin and different, chemically, from the direct reacting bilirubin. They support this view by the observation that direct reacting serum, when mildly oxidized with 0.3 per cent hydrogen peroxide exhibits the characteristic behavior of indirect serum bilirubin. It is difficult to understand, however, how the addition of alcohol to this "oxidized bilirubin" could reduce the pigment again to give the direct reacting type.

Ramsay (145) has observed large increases of serum bilirubin in horses after fasting for forty-eight hours.

Hawkinson *et al.* (146) have described a modification of Harrison's test for bilirubin in the urine, especially suitable for mass and serial usage (principally for the early detection of infectious jaundice): strips of filter paper impregnated with barium chloride dipped into urine containing bilirubin and then treated with ferrichloride show the green color of biliverdin. A standard color chart allows the semiquantitative utilization of this test (147) [for the methylene blue test of bilirubin see (148)].

According to analyses of With (149) the daily bilirubin output of patients with bile fistula is about 200 to 400 mg., with variations between 50 and 900 mg; the daily excretion of urobilinoids in the feces lies between 25 and 250 mg. in normal man. In patients with pronounced jaundice, the sweat, tears, saliva and gastric juice were

found to be free of bilirubin; traces of this pigment could be found, in some cases, in cerebrospinal and eye liquors (150).

With (151) has discussed the various ways of determining the total bilirubin production in man: measurement of bilirubin excretion with the bile, of urobilinoid excretion in the feces, of excretion of bilirubin in jaundiced patients, and of serum bilirubin concentration. Different and independent series of measurements show considerable individual variations of the bilirubin production which are not yet clearly understood. In extreme pathological cases (complete biliary obstruction, acute yellow liver atrophy without jaundice) practically all the hemoglobin seems to be broken down to the dipyrromethenes bilifuscin and mesobilifuscin, only in significant amounts of bilirubin being formed.

Human serum contains yellow substances different from bilirubin and the carotenoids (corresponding to about 8 mg. of bilirubin per 100 cc. of serum) which With (152, 153) believes to resemble bilifuscin and mesobilifuscin or the so-called xanthorubin, a yellow compound present in the serum of hepatectomized dogs. Lups & Meijer (154) describe pigments of jaundiced sera which do not give the diazo reaction.

Urobilinogens.—Schwartz *et al.* (155) have shown that both urobilinogens (mesobilirubinogen and stercobilinogen) give the same color with the Ehrlich aldehyde reagent and described a quantitative method of determination of urobilinogens in urine and feces. Petroleum ether extraction separates the urobilinogens from other Ehrlich reacting substances, probably nonurobilinogen in character. Watson *et al.* (156, 157) have simplified the procedure for clinical use. Maclagan (158) and Kelly *et al.* (159) have determined fecal and urine urobilinogens. Stich (160) has found stercobilin to be a physiological constituent of urine.

Biliverdin.—Tixier (13) has described the chromatographic purification of the methyl ester of biliverdin IX α and has isolated this pigment from the egg shells of the emu (*Dromiceius Novae Hollandiae*).

Bile pigments in invertebrates.—Lederer & Hutterer (161) have studied the pigments of the violet secretion of the sea hare (*Aplysia punctata*); there are at least two chromoproteids, the prosthetic groups of which resemble mesobiliviolin and mesobilierythrin. Their origin from the chromoproteins of red algae is probable.

Tixier (13) describes two new blue bile pigments, the amorphous helioporobilin isolated from the calcarious skeleton of the blue coral *Heliopora caerulea*, and turboglaucobilin (m.p. 206°, tentative formula: $C_{37}H_{40}O_{12}N_4$) from the shells of the mollusc *Turbo Regenfussi*, a pigment which resembles coproglaucobilin (162).

Okay (163) has described blue and red chromoproteins of Orthoptera, containing bile pigments as prosthetic groups.

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THE TERPENES (IN RELATION TO THE BIOLOGY OF GENUS *PINUS*)

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INTRODUCTION

Chemistry of essential oils, or more specifically chemistry of terpenes, has advanced a great deal, owing to the efforts of Wal-lach, Tchirch, Ruzicka, Kremers, and more recently Simonsen and his co-workers, and Haagen-Smit and his associates.

On the contrary, the application of the chemistry of essential oils to the problems of biology has been utterly neglected and very little organized work has been done in this direction. A notable exception is, of course, the classical research on the Eucalypts and their essential oils by Baker & Smith (6)² whose work was supple-mented by more recent and very accurate studies by Penfold & Morrison (30).

Baker & Smith (4) point out that "a remarkable constancy is found in the chemical constituents of the oil of any particular spe-cies (of Eucalypts) wherever grown," and that this constancy is of great value in distinguishing between the several species. They concluded that the chemical composition may safely be used to distinguish between species, especially when morphological char-acters are not clear.

Baker & Smith also studied Australian conifers (5). Here again they considered chemistry of leaf oil as an aid in identifying and classifying different species. Discussing the distribution of dif-ferent terpenes among seventeen species of the genus *Callitris*, Baker & Smith stated that "although the constituents in all the oils appear to be the same they vary in amount in each well-de-fined species" and are comparatively constant, so that "each spe-

¹ Maintained by the Forest Service, U. S. Department of Agriculture, in co-operation with the University of California, Berkeley, California.

² Baker & Smith divided two hundred odd species of Eucalypts into eight groups, each having a different composition of essential oil. Their group 8 is very artificial. In this group are lumped all species that have essential oils of un-usual composition.

cies has its own characteristic oil, and the determination of the amount of its chemical constituents is sufficient to indicate its origin in most cases."

Numerous investigations by Baker & Smith demonstrated clearly that volatile oil obtained from the foliage of many trees of a species is constant in its composition. For instance, they found that "constituents of the oil of *Eucalyptus globulus* are practically constant from whatever locality the material is obtained." The oil distilled from material collected in New South Wales differed in no respect from the product of trees growing in Tasmania or in Victoria. *Eucalyptus cneorifolia* oil analyzed in 1891 in England had an optical rotation of -3.53° and density 0.923¹⁶; a sample of oil from the same species analyzed in 1901 gave a rotation of -3.0° and density 0.929¹⁵.

Eucalyptus sideroxylon oils were examined by Baker & Smith with the following results:

Source of material	Angular rotation	Per cent of Cineol
Near Sydney, Dec. 1900	$+1.90^\circ$	60
Condobolin, March 1901	$+3.19^\circ$	57
Narrabry, July 1901	$+1.39^\circ$	58

The three localities are about three hundred and fifty miles apart.

In general, they found that, with the exception of perhaps half a dozen, all species possess comparatively constant morphological and chemical characters throughout their geographic distribution.

McNair (25) analyzed Baker & Smith's work very carefully and reported that there is not always a correlation between taxonomy and chemistry of the essential oils.

Weevers (46) is of an opinion that difficulties in reconciling biochemistry and taxonomy are encountered because there has been a tendency to correlate a single chemical substance with the taxonomic position of a plant; if not a single chemical substance, but a combination of chemical substances is considered, a correlation between biochemistry and taxonomy would be found.

Penfold & Morrison (30) contributed a great deal to the solution of taxonomic problems by studying essential oils of Eucalypts.

Within *Eucalyptus dives*, besides the laevorotatory "type" rich in piperitone and in phellandrene, Penfold & Morrison established three other forms whose essential oils varied considerably in composition. It appears from their research that these variations in the composition of essential oil were not caused by environmental factors but rather by the hereditary traits of the trees.

Miller (26) studied very intensively the genus *Pycnanthemum* and its volatile oils. He found that the optical property of composed samples of *P. tulliana* oil remained practically the same during all nine years of the investigation.

It is then evident that in some plants composition of the essential oil is quite stable. As shown by Penfold & Morrison (30), qualitative variations in the composition of volatile oils could be traced to the existence of different stable forms within the species.

The foregoing studies seem to indicate that when a problem of validity of essential oils, as a taxonomic characteristic of a species, is approached in a systematic way, and when a taxonomic unit (be it a complex species or a genus) is thoroughly studied, a great deal could be learned as to how the composition of essential oil is related to the taxonomic position of a plant. With these thoughts in mind the author will attempt to discuss the present status of our knowledge of turpentines of the genus *Pinus*, and their possible significance in biological sciences.

CURRENT KNOWLEDGE OF PINE TURPENTINE

Oleoresin or gum, as it exudes from a wounded conifer tree, consists of volatile oils, turpentine, and nonvolatile residue, rosin. Volatile or essential oil is a generic term that includes, besides gum turpentines, many different oils chiefly used in perfumery. Turpentine is a common trade name of a mixture of volatile oils, generally obtained by steam distillation of oleoresin of pines. The turpentine is chiefly composed of terpenes, monocyclic and bicyclic; the occurrence of sesquiterpenes is not infrequent, but oxygenated compounds are rare. Paraffin hydrocarbons are found in some turpentines.

Genus *Pinus* consists of some eighty-four species. The taxonomy of the genus has not yet been settled; some new species may be added in the future, or some existing species may be lumped together. There are some groups that need revision. There may be several varieties in some species, and there are some unknown

pinus that still await description. The most recent monograph on the genus *Pinus* was written in 1914 by Shaw (32), and his system of classification is used in this review. Shaw lists sixty-six species; a few pines whose turpentines have been analyzed are included in the review even though they are not listed by Shaw as valid species.

Advantages of using turpentine in a biological study are several. Turpentine can be obtained easily in large enough quantity to permit its thorough analysis; variations in properties of turpentine in individual trees can be easily ascertained. Turpentines of many species of pine have already been analyzed by reliable workers. The genus *Pinus* appears to be very well fit for a biochemical study of this kind. It is not too large, and yet not too small; its turpentines are much simpler than volatile oils obtained from foliage (as the essential oils of Eucalypts). There occurs within the genus a moderate degree of natural hybridization, so composition of turpentines in hybrids can be studied. Several man-made hybrids facilitate a study of this character.

CONTRIBUTORS TO KNOWLEDGE OF TURPENTINES

American commercial turpentine, which is a product of *Pinus caribaea* and *P. palustris*, has been studied intensively by American workers (9, 29).

Simonsen (39 to 43) and his co-workers in India have analyzed turpentines of several Asiatic pines. Although Simonsen's interest in the subject was technological and chemical, rather than biological, he contributed a great deal to the understanding of the distribution of different constituents of turpentine within the genus *Pinus*. He discovered Δ^3 -carene, which has since been found to be an important ingredient of the most common Eurasian pine, *Pinus sylvestris*, and also of some Asiatic and western American pines. He discovered the presence of n-undecane ($C_{11}H_{24}$) in turpentine of *P. excelsa*, and he ventured to say that closely related pines contain closely related terpenes.

Schorger (34, 35, 37) investigated nine species, reported phellandrene and cadinene for the first time for pines, and verified the presence of n-heptane (C_7H_{16}), reported by previous investigators, in turpentine of *Pinus jeffreyi* and *P. sabiniana*.

French workers (45) have studied intensively turpentine of *Pinus pinaster*, and to a lesser extent the turpentine of *P. halepensis*.

Vèzes & Dupont (45) contributed a great deal to the develop-

ment of physical methods of analyzing turpentine. Darmois (10) achieved interesting results in studying rotatory dispersion of turpentine. Darmois' method was used by Arbuzov in the analysis of *P. sylvestris* turpentine (1), and later by Palkin in the analyses of American (i.e., *P. palustris*, *P. caribaea*) turpentine (29). It appears from these studies that rotatory dispersion is of a particular diagnostic significance in studying properties of turpentine.

A great deal of information regarding the composition of turpentine came from scattered reports of individual workers interested in one or another pine, chiefly as a source of commercial turpentine. The Japanese investigated the chemistry of *Pinus thunbergii* (38) and *P. taiwanensis* (22). De Santos, West & Fontanoza (11) studied turpentine of *P. insularis*. Foote & Mirov (17) investigated the turpentine of *P. monticola* obtained in California. Mirov analyzed turpentine of two California pines, *Pinus coulteri* (27) and *P. muricata* (28).

As these studies progressed it became more and more evident that the problem of turpentine studies in genus *Pinus* had to be organized more systematically. If their biological application was to be sought, more attention had to be paid to the biological source of turpentine, i.e., to the tree from which it is obtained; comparable methods of oleoresin extraction from the trees had to be used; and standardized methods of separating turpentine from rosin had to be employed.

AVAILABLE INFORMATION

Information is still lacking on the composition of turpentine of some pines, such as *P. peuce* of Greece, *P. bungeana* of China, *P. parviflora* of Japan, *P. cembra* of Europe, or *P. balfouriana* of California. The *Pinus cembroides* complex, composed of several closely related and probably easily intercrossing species (*P. edulis*, *P. monophylla*, *P. quadrifolia*, *P. cembroides*), has been only partly studied. Although in Mexico turpentine operations have been conducted for some time on a large scale, no information is available regarding the chemistry of any of the Mexican species of pines. Turpentine of several North American and Asiatic pines have not been investigated.

Despite this incomplete knowledge, considerable is known. Research with turpentine started with the three species *P. palustris*, *P. caribaea* of southeastern United States, and *P. pinaster* of France. Chemical composition of turpentine of these three

pinene is very similar and very simple: 90 per cent or more of it consists of a mixture of α -pinene and β -pinene. When other species of pines were investigated it was found that not all turpentine is as simple as those of the three pines mentioned above. Available information is summarized in Table I (pp. 521-30), arranged into two sections and thirteen groups according to Shaw (32). The following deductions can be made from information in that table.

Most of the pines contain pinene. Only *P. jeffreyi*, *P. sabiniana*, *P. torreyana*, and possibly *P. murrayana* turpentine are completely devoid of pinene. α -Pinene, the most common terpene among pines is found in two modifications, dextrorotatory and laevorotatory. β -Pinene is not as common as α -pinene; it is found chiefly in a laevorotatory form. The dextrorotatory β -pinene is found only in turpentine of Asiatic pines: *P. gerardiana*, *P. merkusii*, and *P. khasya*.

Limonene is found in many pines and is always of a laevorotatory form. An optically inactive form of limonene, dipentene, is very seldom found in gum turpentine, and only in small quantity.³ *P. serotina* turpentine contains 80 to 90 per cent of limonene; *P. pinea*, 75 per cent; *P. torreyana*, 75 per cent.

Phellandrene (*l*-form) so far has been found in two pines of western America: *P. murrayana* turpentine consists almost entirely of β -phellandrene; *P. coulteri* contains about 20 per cent of this terpene.

Δ^3 -Carene was found to the extent of 10 to 38 per cent in four Old World pines. Recently it was found in turpentine of *P. albicaulis*, *P. ponderosa*, and *P. washoensis* of the western United States.

Camphene is found in small quantities on turpentine of several pines. In *P. clausa* turpentine, however, there is as much as 10 per cent of this terpene.

Generally speaking, sesquiterpenes ($C_{15}H_{24}$) are found in small quantity in turpentine of many pine species. Cadinene is so far the most frequently found sesquiterpene in pine turpentine. It occurs in *P. edulis*—as much as 15 to 20 per cent; in *P. monophylla*, 3 to 6 per cent; in *P. ponderosa*, 10 per cent. Longifolene, a sesquiterpene first discovered by Simonsen in *P. longifolia* of India, was later found in *P. merkusii* of S. W. Asia, *P. taiwanensis* of Formosa, and recently in *P. torreyana* of California.

³ Turpentine obtained by steam distillation of pine stumps contains considerable amounts of dipentene.

TABLE I
COMPOSITION OF TURPENTINE OF PINES
SECTION HAPLOXYLON—SOFT (WHITE) PINES
SUBSECTION CEMBRA

Name	Native range	Composition of turpentine	Reference
Group Cembrae <i>P. sibirica</i> Mayr	N.E. Europe and Siberia	Only partially analyzed. Old oxidized gum was used; only 6% turpentine was obtained. Turpentine contained presumably 20% <i>d</i> - α -pinene. Specific gravity 0.865 (15°C.).*	(31)
<i>P. albicaulis</i> Engelm.†	Western U. S.	35% Δ^1 -carene 10% other terpenes 7% α sesquiterpene 30% α diterpene	(19)

Of group Cembrae the following species have not been investigated: *P. koraiensis*, *P. pumila* (N.E. Asia); *P. cembra* (Europe)

Group Flexilis, consisting of *P. flexilis* and *P. armandi*, has not been investigated.

Group Strobi <i>P. lambertiana</i> Dougl.	Oregon to Lower California	70-75% <i>d</i> - α -pinene 5% β -pinene 2-3% possibly phellandrene 10-12% sesquiterpene; undecane suspected	(34)
<i>P. griffithii</i> McClelland (<i>P. excelsa</i> Wall.)	Himalayas	88% <i>d</i> - α -pinene; small quantities of <i>d</i> -terpineol; n-undecane; sesquiterpene	(40)
<i>P. monticola</i> Dougl.	Western U. S.	66% <i>d</i> - α -pinene 26% β -pinene 1-2% n-undecane; sesquiterpene	(16)
<i>P. strobus</i> L.†	Eastern U. S.	80% <i>dl</i> - α -pinene 10% <i>l</i> - β -pinene	(19)

Of group Strobi, the following species have not been investigated: *P. ayacahuite* (Mexico), *P. parviflora* (Japan), and *P. peuce* (Balkans)

Group Cembroides <i>P. monophylla</i> Torr. & Frém.	W. U. S. and Lower Calif.	85% <i>d</i> - α -pinene 4-5% <i>dl</i> or <i>l</i> -limonene 4-6% sesquiterpene-cadinene	(35)
<i>P. edulis</i> Engelm.	Colo., Utah, W. Okl., Tex., N. M., Ariz., North Mexico	70-75% <i>d</i> - α -pinene 5% β -pinene 15-20% sesquiterpene-cadinene	(34)

Of group Cembroides, the following species have not been investigated: *P. pinceana*, *P. nelsonii*, *P. cembroides* of Mexico, and *P. quadrifolia* of southwestern U. S.

* Incomplete information.

† Analysis not completed.

TABLE I—(continued)

Name	Native range	Composition of turpentine	Reference
Group Gerardianae <i>P. gerardina</i> Wall.	N.W. Himalayas	73 % <i>d</i> - α -pinene 7 % <i>d</i> - β -pinene sesquiterpene, sesquiterpene-alcohol (small quantity). Fractions bet. 170° and 250°C. at 705 mm. amounting to 1.3% did not contain dipentene, phellandrene, terpinene or terpineol.	(42)
<i>P. bungeana</i> Zucc.	Hupeh, China	Not known	
Group Balfourianae, consisting of <i>P. balfouriana</i> (Calif.) and <i>P. aristata</i> (W. U. S.), has not been investigated.			

SECTION DIPLOXYLON—HARD PINES

SUBSECTION PARAPINASTER

Name	Native range	Composition of turpentine	Reference
Group Leiophyllae, consisting of <i>P. leiophylla</i> and <i>P. lumholtzii</i> , both of Mexico, has not been investigated.			
Group Longifoliae <i>P. roxburghii</i> Sarg. (<i>P. longifolia</i> Roxb.)	Foothills of Himalayas	24.8 % <i>l</i> - α -pinene 37.6 % Δ^4 -carene 9 % <i>l</i> - β -pinene sesquiterpene-longifolene	(39)
<i>P. canariensis</i> Smith	Canary Islands	94 % <i>dl</i> - α -pinene 2 % <i>l</i> -limonene 1.5 % bornyl acetate, cadinene	(19)
Group Pineae <i>P. pinea</i> L.	Mediterranean Region	75.4 % <i>l</i> -limonene 16.7 % <i>l</i> - α -pinene 6.6 % sesquiterpene	(15)

SUBSECTION PINASTER

Name	Native range	Composition of turpentine	Reference
Group Lariciones <i>P. khasya</i> Royle	Burma, India	70 % <i>d</i> - α -pinene 10 % <i>d</i> - β -pinene 10 % <i>d</i> -longifolene	(41)
<i>P. pithyusa</i> Steven	Caucasus	69.8 % <i>l</i> - α -pinene 23.6 % Δ^4 -carene 1 % unidentified component boiling at 73° to 78° at 14.5 mm. of pressure, 5 % high boiling fraction.	(3)
<i>P. sylvestris</i> L.	Eurasia	76 % <i>d</i> - α -pinene 14 % <i>d</i> - Δ^4 -carene 7 % unidentified laevorotatory terpene 1 % higher fractions	(2)

TABLE I—(continued)

Name	Native range	Composition of turpentine	Reference
<i>P. thunbergii</i> Parl.	Japan	73% <i>l</i> - α -pinene Small amount of camphene 15% tricyclic sesquiterpene	(38)
<i>P. nigra</i> Arnold	So. Europe and Asia Minor	96% <i>l</i> - α -pinene 1% limonene 1% sesquiterpene Trace of esters	(13)
<i>P. merkusii</i> DeVriese	Philippines, Sumatra Burma, Indo-China	78% <i>d</i> - α -pinene 7% β -pinene 10% <i>d</i> - Δ^1 -carene 5% <i>d</i> -longifolene	(43)
<i>P. insularis</i> Endl.	Philippines and Burma	Only pinene was identified by preparing pinene hydrochloride.†	(11)
<i>P. taiwanensis</i> Hay	Formosa	73% <i>l</i> - α -pinene 13% <i>d</i> -longifolene Dipentene, small quantity Bornyl acetate, small quantity	(22)
Of group Lariciones, the following species have not been investigated: <i>P. resinosa</i> (E. U. S.), <i>P. tropicalis</i> (Cuba), <i>P. massoniana</i> (China), <i>P. densiflora</i> (Japan), <i>P. montana</i> (So. Europe), <i>P. luchuensis</i> (Luchu Islands), <i>P. sinensis</i> (China).			
Group Australes			
<i>P. palustris</i> Mill.	SE. U. S.	64.3% <i>d</i> - α -pinene 31.8% β -pinene .07% heads 0.7% tailings‡	(9, 14)
<i>P. caribaea</i> Morelet	S.E. U. S. Honduras and Guatemala	75.6% <i>l</i> - α -pinene 21% β -pinene 3.2% tails	(14)
<i>P. taeda</i> L. §	S.E. U. S.	75-80% <i>d</i> - α -pinene 9-12% β -pinene	(19)
<i>P. echinata</i> Mill.	S.E. U. S.	Some pinene, mostly limonene*	(20)
<i>P. jeffreyi</i> Grev. & Balf.	S. Ore., Calif., Lower Calif.	95% n-heptane n-octyl, n-nonyl n-decyl aldehydes	(16, 35)
<i>P. ponderosa</i> Laws.	W. U. S. and adjacent parts of Mexico	5% <i>l</i> - α -pinene 60% <i>l</i> - β -pinene 20% limonene 10% cadinene	(34)
<i>P. ponderosa</i> var. <i>scopulorum</i> Engelm.	Rocky Mt.	60-70% <i>d</i> - α -pinene 20% <i>l</i> - β -pinene 20-25% limonene	[(34) p. 15]

† Analysis not completed.

‡ In tailings are found: dipentene, terpinolene, bornyl acetate, methyl chavicol.

§ Analyzed by T. H. Wang, Calif. Inst. of Technology.

* Incomplete information.

|| Ponderosa pine complex should be studied in detail. Recently analyzed sample (23) consisted of a mixture of 20% *l*- Δ^1 -carene, 45% *l*- β -pinene and 3% limonene.

TABLE I—(continued)

Name	Native range	Composition of turpentine	Reference
<i>P. washoensis</i> Mason & Stockwell	W. Nevada	Varying mixture of Δ^4 -carene and <i>l</i> - β -pinene	(19)
Of group <i>Aurales</i> , the following species have not been investigated: <i>P. pseudostrobus</i> , <i>P. montesumae</i> , <i>P. leucote</i> , <i>P. lawsonii</i> of Mexico; <i>P. occidentalis</i> of West Indies, <i>P. glabra</i> of S.E. U. S., <i>P. ponderosa</i> var. <i>arizonica</i> (Engelm.) Shaw and <i>P. latifolia</i> Sarg. (<i>P. apachea</i> Lemm.) of Arizona.			
Group <i>Insignis</i> <i>P. halepensis</i> Mill.	Mediterranean Region	95 % <i>d</i> - α -pinene 3.8 % sesquiterpene 1 % bornyl acetate	[(45) p. 297]
<i>P. pinaster</i> Ait.	France	62.8 % <i>l</i> - α -pinene 26.8 % <i>l</i> - β -pinene 10 % higher boiling fractions.	[(45) p. 293]
<i>P. clausa</i> (Engelm.) Vasey	E. U. S.	10 % <i>l</i> - α -pinene 10 % <i>l</i> -camphene 75 % <i>l</i> - β -pinene	(36)
<i>P. rigida</i> var. <i>serotina</i> (Michx.) Loud. (<i>P. serotina</i> Michx.)	S.E. U. S.	80–90 % <i>l</i> -limonene	(21)
<i>P. banksiana</i> Lamb.	N.E. U. S., Canada	85 % <i>d</i> - α -pinene 10 % β -pinene	(19)
<i>P. contorta</i> var. <i>latifolia</i> Engelm. (<i>P. murrayana</i> Grev. & Balf.)	N.W. U. S., Canada	Almost entirely <i>l</i> - β -phellandrene	(34)
<i>P. muricata</i> D. Don	Calif.	Mostly <i>d</i> - α -pinene Less than 1 % camphene	(28)
<i>P. attenuata</i> Lemm.	Calif., So. Oregon	Over 95 % <i>d</i> - α -pinene	(19)
<i>P. radiata</i> D. Don	Calif.	65 % <i>d</i> - α -pinene 30 % β -pinene 1 % limonene	(19)
Of group <i>Insignis</i> , the following species have not been investigated: <i>P. gringlei</i> , <i>P. occarpa</i> , <i>P. greggii</i> , <i>P. patula</i> of Mexico; <i>P. virginiana</i> , <i>P. rigida</i> , <i>P. pungens</i> of E. U. S.; <i>P. contorta</i> and <i>P. remorata</i> , Pacific Coast of U. S.			
Group <i>Macrocarpae</i> <i>P. torreyana</i> Parry	Calif.	75 % <i>l</i> -limonene 4 % longifolene 10 % <i>n</i> -decyl aldehyde .02 <i>n</i> -lauryl aldehyde .02 <i>a</i> C ₁₁ carbonyl compound 5 % <i>n</i> -undecane	(18)
<i>P. coulteri</i> D. Don	Calif. and Lower Calif.	30–35 % <i>l</i> - α -pinene 35–45 % <i>l</i> - β -phellandrene 10 % <i>n</i> -undecane 5 % <i>n</i> -heptane	(27)
<i>P. sabiniana</i> Dougl.	Calif.	95 % <i>n</i> -heptane. Small amounts of <i>n</i> -octyl, <i>n</i> -nonyl, <i>n</i> -decyl, and <i>n</i> -myristic aldehydes	[(44), (34) p. 21]

Except for certain aldehydes found in appreciable quantity (5 to 10 per cent) in turpentines of *P. torreyana*, *P. sabiniana*, and *P. jeffreyi*, oxygenated compounds are extremely rare in pine turpentines. The only alcohol found, terpineol, occurs in one species, and in very small quantity, *P. excelsa* of India. Of esters, the only one so far identified has been reported as bornyl acetate. It is found in very small quantities in *P. canariensis*, *P. taiwanensis*, *P. halepensis*, and traces of it were found in the tailings of *P. palustris*. Traces of esters were reported in *P. nigra*.⁴

As to the paraffin hydrocarbons, n-heptane is found in turpentine of *P. jeffreyi*, *P. sabiniana*, *P. coulteri*, and *P. torreyana*. In the first two pines 95 per cent of the turpentine consists of n-heptane and no terpenes are found in these species. In *P. coulteri* n-heptane is found in small quantity. In *P. torreyana* less than 0.1 per cent was found. Normal undecane ($C_{11}H_{24}$) was found in small quantities in *P. excelsa*, *P. monticola*, *P. coulteri*, and *P. torreyana*; a paraffin hydrocarbon reported in *P. lambertiana* by Schorger is probably also n-undecane. A trace (less than 0.1 per cent) of nonane is found in Torrey pine turpentine.⁵

As to the geographic distribution of terpenes among the species of genus *Pinus*, the eastern United States pines have relatively very simple turpentines composed chiefly of terpenes. No sesquiterpenes, or paraffin hydrocarbons have been recorded in those pines, and oxygenated compounds were found only in minute quantity in tailings of *P. palustris* turpentine. In the simplicity of their turpentines, eastern United States pines resemble western European pines, although small amounts of sesquiterpenes have been reported in two of the European pines.

P. sylvestris, growing in the old world from the Atlantic to the Pacific and from Norway to Spain, differs from the rest of the European pines in that besides pinene it contains about 15 per cent of Δ^3 -carene. This species may be considered as a biochemical link between western European and southeastern Asiatic pines.

Chemistry of turpentines of the western United States pines is rather complicated. There are on the Pacific Coast some pines (*P. muricata*, *P. attenuata*) possessing simple turpentines, but many

⁴ In tailings of *P. palustris* turpentine, Chadwick & Palkin (9) found minute quantities of many substances not listed above (see Table 1).

⁵ Previously n-nonane was found in volatile oil of *Sarothra gentianoides*. Marion (24).

of the western pines so far examined contain in their turpentines: aldehydes, considerable amounts of sesquiterpenes, and paraffin hydrocarbons. In their complexity they resemble some pines of southeastern Asia.

APPLICATION OF CHEMISTRY OF TURPENTINES TO BIOLOGY

As information on chemical composition of different turpentines gradually accumulated, some attempts were made to understand the biological significance of differences in their chemical composition. Dupont (12) attempted to classify pines into α - and β -pinene pines; Simonsen (41) noted that "the presence of the same sesquiterpene (longifolene) in *P. Khasya* as in *P. longifolia* is of considerable interest in view of the close botanical relationship of these two pines."

Schorger (33) was the first to point definitely towards the relation of turpentine chemistry to the taxonomical position of a pine. He found that, generally, composed samples of *P. ponderosa* from California are laevorotatory and that they consist mainly of β -pinene, while the oil from *P. ponderosa* var. *scopulorum* is dextrorotatory and consists largely of α -pinene. This decided difference in chemistry of turpentines of the two pines appeared to Schorger as a valid reason for considering them as two separate taxonomic entities.

Schorger investigated turpentines of *P. ponderosa* and *P. jeffreyi* (which is considered by many botanists a variety of *P. ponderosa*) and also intermediate forms that in the field looked like Jeffrey pines and were assumed to be crosses between the two. After turpentines of these pines were analyzed, it was found that the typical ponderosa pines had laevorotatory turpentines (chiefly β -pinene and limonene); the Jeffrey pines were in all cases heptane-yielding pines (no terpenes). But out of five "cross varieties," four turned out to be typical β -pinene-limonene pines and one yielded turpentine composed almost entirely of *l*-limonene. One sample of turpentine came from a peculiar tree, appropriately named "bastard pine" by the local foresters because it did not look like the general run of ponderosa pine. Its turpentine proved to be dextrorotatory and contained α -pinene instead of the common β -pinene. Schorger concluded that this dextrorotatory α -pinene variety of ponderosa pine coincided with the botanical variety *scopulorum*, a Rocky Mountain form of ponderosa pine.

In some localities *P. jeffreyi* and *P. ponderosa* grow together. While it is not difficult to differentiate between Jeffrey pine and typical ponderosa pine, there are varieties of ponderosa pine that externally look very much like Jeffrey pine: ponderosa pine is quite variable and sometimes difficult to identify. The chemistry of Jeffrey pine turpentine has been studied intensively and it was found that presence of the aldehydes in this pine and absence of the aldehydes in ponderosa pine is a very reliable way to tell these two pines apart. In dubious cases, foresters distinguish these two pines by difference of odor emanated by the trees, i.e., by the chemical difference.

Because *P. jeffreyi* and *P. ponderosa* morphologically look alike, they have been considered to be very closely related. Biochemically, however, *P. jeffreyi* is more closely related to the group *Macrocarpae* than to the group *Australes*. Heptane has been found in turpentes of all three pines comprising the *Macrocarpae* group, and it was also found in *Pinus jeffreyi*. Similar aldehydes are found in *P. jeffreyi* and in the pines of group *Macrocarpae*. Jeffrey pine crosses with both *P. ponderosa* and *P. coulteri* in nature, perhaps more fully with the latter.

It is extremely difficult to differentiate morphologically between *P. strobus*, of northeast, and *P. monticola* of northwest United States. Biochemically, however, there is no difficulty in distinguishing the two species: *P. monticola* contains n-undecane in its turpentine; *P. strobus* turpentine contains no n-undecane.*

P. pithyusa is considered by Shaw as being of the same species as *P. halepensis*, yet the biochemical characteristics of *P. pithyusa*, compared with those of *P. halepensis*, indicate that these two pines do not belong to the same species. While *P. halepensis* turpentine contains about 95 per cent *d*- α -pinene, *P. pithyusa* contains 70 per cent *l*- α -pinene and 24 per cent *d*- Δ^3 -carene, which places it closer to *P. sylvestris* than to *P. halepensis*. Some botanists do not consider *P. pithyusa* as a form of *P. halepensis*, but rather as an independent species.

In Shaw's monograph on pines there is a small group of pines called *Longifoliae*. It consists of two species, *P. longifolia* of India and *P. canariensis* growing on the Canary Islands. These two pines

* *P. monticola* turpentine came from northeastern California. It would be interesting to analyze turpentine of this pine from Montana and Idaho.

resemble each other morphologically, but biochemically they are quite different. Δ^3 -Carene, which is present in *P. longifolia*, is absent in *P. canariensis*. In the latter pine the major component is α -pinene. The sesquiterpenes of the two pines are also different, longifolene is found in the former and cadinene is found in the latter.

"American turpentine" of the world trade is a mixture of turpentine of *P. palustris* and *P. caribaea*. Composed samples of turpentines of the two pines differ in that *P. caribaea* turpentine is laevorotatory because of a predominance of *l*- α -pinene; while turpentine of *P. palustris* is dextrorotatory on account of a predominance of *d*- α -pinene. The minor eastern United States pines have not been analyzed yet, with the exception of *P. serotina*, which was found to contain 80 to 90 per cent of limonene in its turpentine.

Three closely related pines of the group *Insignis*, *P. muricata*, *P. attenuata*, and *P. radiata*, also have very similar turpentines, as seen from the following tabulation:

Species	Composition of turpentine
<i>P. muricata</i>	<i>d</i> - α -pinene 98%; camphene less than 1%
<i>P. attenuata</i>	<i>d</i> - α -pinene 98%
<i>P. radiata</i>	<i>d</i> - α -pinene 70%; <i>l</i> - β -pinene 25%

There are some pines in the group *Insignis*, such as *P. pinaster*, *P. halepensis*, and *P. banksiana*, whose turpentines also are composed of pinene (*P. halepensis*—95 per cent α -pinene; *P. pinaster* 63 per cent α -pinene 26.8 per cent β -pinene, *P. Banksiana* 90 per cent α -pinene). But *P. murryana*, which belongs to the same group, has very different turpentine. It consists almost entirely of *l*- β -phellandrene. Another pine in the same group, *P. serotina*, has turpentine almost entirely composed of *l*-limonene.

Turpentines of four of the seven pines of the group *Strobi* have been examined. Of these four, two western United States pines (*P. lambertiana* and *P. monticola*), and one Himalayan pine (*P. excelsa*) were found to contain *n*-undecane ($C_{11}H_{24}$) and an abundance (60 to 88 per cent) of dextrorotatory α -pinene. The fourth pine analyzed, *P. strobus* of the northeastern United States apparently does not contain in its turpentine any *n*-undecane but is composed of *d*- α -pinene and *l*- β -pinene. Its optical activity $[\alpha]_D^{20.5}$ is -0.87 .

P. albicaulis was the first rare, high-elevation white pine whose turpentine was analyzed very recently. The composition of its turpentine is rather unusual: it contains a large quantity of *d*- Δ^8 -carene. Previously carene was found in several pines of Europe and Asia. There is also a large amount of an apparently new diterpene in *P. albicaulis* turpentine.

Several white pines in the Haploxylon Section are lumped by some botanists into one unit called *P. cembroides*. Other botanists separate them into at least four species (*P. cembroides*, *P. quadrifolia*, *P. monophylla*, and *P. edulis*). The taxonomy of these pines is very confusing—apparently some of them cross in nature. Turpentines of two of them have been studied by Schorger. *P. monophylla* and *P. edulis* both are rich in α -pinene; both are dextrorotatory. *P. monophylla* has 4 to 6 per cent cadinene and *P. edulis* contains 15 to 20 per cent of cadinene. It would be of interest to analyze turpentines of the rest of these pines, i.e., *P. cembroides* and *P. quadrifolia*, as well as turpentines of the closely related Mexican pines, *P. pinceana* and *P. nelsoni*.

VARIAION OF COMPOSITION OF TURPENTINE WITHIN A SPECIES

Available data (see Table 1) indicate that the composition of pine turpentine obtained from a composed sample of oleoresin is specific. Turpentine of *P. jeffreyi* always has produced n-heptane and that of *P. pinea* always contained limonene. A composed sample of *P. caribaea* always yielded a laevorotatory mixture of α - and β -pinene, while *P. palustris* would always give a dextrorotatory mixture of these two terpenes. These are characters presumably valid for these species of pines. Further investigations will show if such specificity would hold true for all species of pines.

However a species does not consist of one organism; it is composed of a population of individual trees. Moreover, a species is a dynamic rather than a static unit, sometimes rather artificial, delimited by an investigator in order to simplify some taxonomic difficulties, or because there is not sufficient knowledge available in regard to the phylogeny of a species in question. Variations of chemical composition of turpentine within a species, even if these are of quantitative character only, are considerable. The causes of these variations may be several. As a species consists of a population of individuals that are not exactly alike genetically, there are

apt to be individual differences in the composition of turpentine in individual trees. Little is known as to how big these differences are. Apparently the degree of variability of physical and chemical characters of a turpentine is somewhat correlated with the ecological and genetic nature of the species. The endemic species with a limited range of distribution appear to be steady in respect to physical and chemical characters of their turpentines. For instance, *P. torreyana* is an endemic pine, naturally growing on an area of a few square miles not far from San Diego, California. Turpentine obtained from three individual trees of this species showed a remarkable constancy of the physical characters of the turpentine (Table II). The turpentine contains 75 per cent of *l*-limonene.

TABLE II
PHYSICAL CONSTANTS OF TORREY PINE TURPENTINE
FROM THREE DIFFERENT TREES

Source of turpentine		Density ₄ ²⁴	Index of refraction _D ²⁵	Special optical rotation [α] _D ²⁶
Tree #1				
August	Sample	.8366	1.4660	-116.03°
September	"	.8357	1.4660	-115.89
October	"	.8366	1.4658	-115.47
Tree #2				
August	Sample	.8359	1.4658	-121.95
September	"	.8358	1.4654	-119.40
October	"	.8357	1.4655	-121.87
Tree #3				
August	Sample	.8356	1.4658	-115.96
September	"	.8355	1.4650	-119.41
October	"	.8360	1.4654	-121.13

Another example of constancy of physical characters is *P. merkusii*, growing in Burma and in Dutch East Indies (8). Turpentine of this pine, which consists chiefly of *d*-α-pinene, has been considered of commercial importance and was studied by many investigators. The physical constants of this pine are given in Table III.

TABLE III

PHYSICAL CHARACTERS OF *Pinus merkusii* TURPENTINE

Origin of turpentine	Density d	Index of refraction n_D^{20}	Specific rotation $[\alpha]_D^{20}$
Burma	.8575 ³⁰ ₁₀	1.4653 ³⁰	+28.67 ³⁰
Dutch East Indies	.8644 ¹⁵ ₁₀	1.4653 ^{25.5}	+36°44' ²⁰
	.8636 ¹⁵ ₁₀	1.4653 ²⁸ ₂₀	+36°50' ²⁸
	.8623 ¹⁵ ₁₀	1.4665 ²⁰	+35.8°
N. Sumatra	.866 ¹⁵ ₁₀	1.468 ²⁰	+35.6°
	.861 ¹⁵ ₁₀	1.470 ¹⁵	+36°26' ^{17.5}
Burma	.8610 ²⁰		+31.45°

This table shows very clearly that *P. merkusii* turpentine collected in different years and at widely separated places possesses very similar physical characters.

On the other hand, a species which occupies a large area and in which botanists are able to distinguish several varieties and forms may be expected *a priori* to have great variations in the chemistry of their turpentines. There would be several varieties corresponding to Penfold & Morrison's physiological forms. At the same time, if a species is not genetically stable and if an infiltration of genes still goes on, individual variations in the turpentines obtained from individual trees probably would be considerable. Variability of turpentine in a large population of a species still awaits an investigation.

In any species one may expect an occasional gene mutation that would affect composition of turpentine of an individual tree. For instance, generally speaking, *P. sylvestris* turpentine is dextro-rotatory and is characterized by the presence of a terpene: Δ^3 -carene. In 1930 two Russian investigators (23) described an unusual turpentine obtained from an individual tree of *P. sylvestris*. It was laevorotatory and was composed of pinene, camphene, and phellandrene. The usual Δ^3 -carene was absent.

When two species of pine are crossed, the chemical characteristics of the turpentine of the hybrid are likely to be more complicated than the turpentines of its parents. Very little is known yet about the inheritance of chemical composition of turpentine in pines. The writer knows of a hybrid between *P. ponderosa* and *P. jeffreyi* that contained in its turpentine both terpenes, inherited

from the ponderosa parent, and heptane from the Jeffrey parent.

Breeding of pines is a new undertaking. Recently several hybrids have been obtained at the Institute of Forest Genetics, and in the future these hybrids will afford an interesting study of inheritance of biochemical characters in their turpentine.

Variations of some physical properties of a turpentine may also depend on the state in which one or another component is found. For instance, α -pinene may be present in the same sample of turpentine in two different modifications: *d* and *l*. It might be possible that fluctuations in rotatory power of turpentine samples obtained from individual trees sometimes are due to variations of optical properties of the same terpene.

When a turpentine consists of an optically inactive compound, the physical and chemical properties of the samples obtained from individual trees are remarkably stable. Out of 12,000 trees of *P. jeffreyi* examined by the writer, only a few were found to deviate from the general run. These few apparently (judging from their morphological characters) were hybrids between Jeffrey pine and ponderosa pine.

Considering all these circumstances, there is little wonder that Black & Thronson (7), studying optical activity of turpentine obtained from six individual trees of *P. caribaea* and six individual trees of *P. palustris* throughout two seasons, found considerable variation of physical characters between the individual trees and between the two seasons. In the turpentine of some individual trees seasonal variation of rotatory power was 3°, in others as high as 8°.

Black & Thronson (7) found that during a season optical properties of turpentine of individual trees of both species do not follow any orderly course. In slash pine turpentine actually changed the sign of optical rotation of its oil from minus to plus for a couple of weeks, but later returned to the original laevorotatory state.

In *P. washoensis* specific rotation of turpentine obtained from nineteen individual trees varied from -9.75° to +10.15°. The method of obtaining oleoresin and the method of extraction were the same in all cases; the variations are probably due to the varying proportions of *d*- Δ^2 -carene and *l*- β -pinene.

Thus it appears that while physical characters within a species do vary, the variations can be traced to such factors as varying

⁷ Unpublished data.

amounts of optical antipodes of the same terpene, varying amounts of two different terpenes commonly found in turpentine of one species, or to hybridization. The whole problem should be studied very carefully, using standardized techniques and a systematic approach. What is already known about the cause of these variations justifies the view that the physical properties of turpentine of a species have definite diagnostic value.

CONCLUSIONS

The attempt has been made to show that a great deal may be learned about phylogeny and taxonomy if a plant group (a genus or species) is analyzed biochemically. It is shown how useful was this approach in the case of the genus *Eucalyptus* and some other plants. The present status of biochemistry of turpentines of the genus *Pinus* has been discussed. Recent investigations have enriched our knowledge of distribution of terpenes in different pines. Although valuable contributions have been made, there are still many species that await investigation. Besides a study of turpentines of different species of pines it seems important to inquire into the variations in composition of turpentines among a population of a species.

It would be a futile task even to attempt to devise a biochemical classification of pines that would replace the existing botanical classification, but at the same time the biochemical characters of a pine may well be used to establish or clarify relationships that are not discernible by morphological characters alone.

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THE ALKALOIDS¹

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Notwithstanding the emergency character of recent alkaloid research several fundamental contributions have been made. Perhaps the most gratifying of these was the total synthesis by Woodward & Doering (1) of *d*-quinotoxine, which in view of Rabe's earlier work constituted a total synthesis of quinine. Noteworthy also were the isolation from *Erythrina* of a series of alkaloidal esters of sulfoacetic acid by Folkers *et al.* (2); the identification of the octahydropyrrocoline ring system in the tertiary veratrine bases by Craig, Jacobs *et al.* (3, 4); the apparent rationalization of the problem of strychnine structure³ by Woodward, Brehm & Nelson (5); and the demonstration of an indole nucleus in quinamine by Kirby (8), in cinchonamine by Raymond-Hamet (9) and by Janot & Berton (10). Problems of isolation, structural elucidation and synthesis do not, however, constitute the whole of alkaloid chemistry. Several new and useful analytical procedures have been developed. Similarly, questions surrounding the origin of the alkaloids in the plants that produce them have begun to receive attention. Finally, interest in the quantitative aspects of biological alkaloid production has been renewed with wartime intensification of demand for drugs and agricultural poisons. Space limitations necessitate restriction of the present discussion to two groups of plant alkaloids in which research has been especially active.

THE CINCHONA ALKALOIDS

Interest in this group has centered about the problem of increasing the supply of quinine and other antimalarials (e.g., totaquine) by actual synthesis and/or biological production.

New records of occurrence.—Exploitation of natural Cinchona

¹ This review covers the period from January, 1944 to December, 1947.

² The author is greatly indebted to Mr. B. A. Krukoff of the New York Botanical Garden for access to the literature on the biological production of the Cinchona alkaloids. Indebtedness is also gratefully acknowledged to Dr. W. G. Frankenburg of the General Cigar Company for the privilege of many stimulating discussions on the subject of tobacco alkaloid chemistry.

³ Robinson has also proposed a new formulation for this alkaloid (6, 7).

stands in Colombia, Peru, Bolivia, and Ecuador has led to partial clarification of the alkaloidal composition of different species complexes. Martin & Gandara (11) described genetic variation within natural populations with respect to the relative proportions of quinine, cinchonine and cinchonidine in the total crystallizable alkaloid complex. Hodge (12) reported new data on the distribution of alkaloids in commercial forms of the four major Peruvian species of *Cinchona*, namely *C. Humboldtiana*, *C. micrantha*, *C. officinalis*, and *C. pubescens*. Steere (13) called attention to the relatively high quinine content of certain strains of *Cinchona pitayensis* and of *Remijia pedunculata*; but Van Leersum (14) long ago showed *pitayensis* to be inferior to the Ledger strains of *Calisaya* for purposes of commercial production, and the history of the utilization of *Remijia* bark is well known (15).

Castillo (16) found large amounts of cupreine in the fruits of *Remijia pedunculata*. Hodge (17) provisionally reported the presence of 3.36 per cent total crystallizable alkaloids in *Ladenbergia malacophylla*, one of the "false Cinchonas."

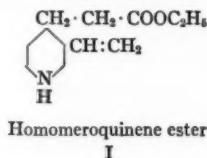
It has unfortunately not been widely recognized that the alkaloids of green *Cinchona* bark (i.e., bark from young twigs) and of *Cinchona* leaves differ greatly in their properties from those of the mature trunk bark. This difference has been emphasized repeatedly by the work of Van Leersum (18), Lotsy (19) and Tatarskaja & Solomko (20). Kerbosch & Van Dorssen (21) as long ago as 1928 showed that the majority of basic materials that are extracted from leaves and green twigs with the usual solvents do not belong to currently recognized groups of *Cinchona* alkaloids and that some of them are apparently glycosidic in nature.

Structural evidence.—Quinamine, one of the minor *Cinchona* alkaloids, can now be regarded with certainty as a derivative of indole (8) and is the first of the *Cinchona* alkaloids to be placed in this category. There is a high degree of probability that cinchonamine (9, 10) may also prove to be a derivative of indole rather than of quinoline. The importance of these facts lies in the observation that the quinuclidine ring structure emerges as the common denominator in *Cinchona* alkaloid chemistry: the quinoline and indole nuclei very likely may be regarded as alternative substituents on the carbinol sidechain. Important implications of this fact are also to be inferred for the field of alkaloid biogenesis.

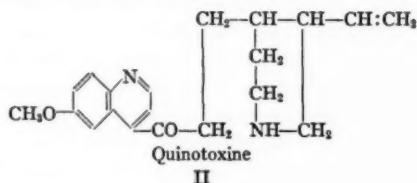
Prelog & Zalán (22) demonstrated the stereochemical relation-

ships between quinine and quinidine and showed that the vinyl groups are *cis* to the C₇-C₈ bridge in the quinuclidine ring. King (23) demethylated hydroquinidine, replaced the hydroxyl with an amino group and removed the latter by diazotization to give dihydrocinchonine. Since cupreine, similarly treated, yielded cinchonidine, it was concluded that the commonly accepted classification of quinine and quinidine and their dihydro derivatives as representing methoxylated derivatives, respectively, of cinchonidine, cinchonine and their dihydro derivatives is justified.

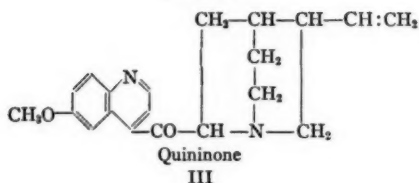
New syntheses.—Prior to 1945 total syntheses among the Cinchona alkaloids had been realized only for members of the group that contain an ethyl rather than a vinyl group attached to the quinuclidine ring. The total synthesis of quinine (and of quinidine) has been accomplished by Woodward & Doering (1) by an elaborate series of steps that appears to have been designed primarily to obtain the vinyl homologue of homocincholoipon ester (ethyl- β -3-ethyl-4-piperidylpropionate), namely homomeroquinene ester (ethyl- β -3-vinyl-4-piperidylpropionate) (I).



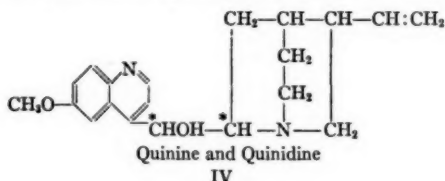
In the final stages of the synthesis, N-benzoylhomomeroquinene ethyl ester was condensed with ethyl quininate to form the corresponding β -keto ester. The latter was hydrolyzed, resolved as the dibenzoyl-*d*-tartrates into *d*- and *l*-quinotoxine (II).



Since Rabe and Kindler had earlier converted *d*-quinotoxine from natural sources into quinone (III) and thence by reduction into a mixture of stereoisomeric alcohols from which quinine and quini-



dine were isolated, the total synthesis of *d*-quinotoxine also constitutes a total synthesis of quinine and quinidine (IV).



(Asterisks mark asymmetric carbon atoms involved in quinine-quinidine and in epiquinine-epiquinidine isomerization.)

Homomeroquinene ester was obtained by the following succession of conversions, some of which appear to be unique: 7-Hydroxyisoquinoline with piperidine and formaldehyde in methanol gave 7-hydroxy-8-piperidinomethylisoquinoline, which on protracted digestion in methanolic sodium methoxide was converted into 7-hydroxy-8-methylisoquinoline. The latter was hydrogenated in acetic acid with platinum to the corresponding 1,2,3,4-tetrahydroisoquinoline. Further uptake of hydrogen was effected only by N-acetylation followed by reduction over Raney nickel at elevated temperature and pressure. From chromic acid oxidation of the reduced N-acetyl compound, *cis*-N-acetyl-7-keto-8-methyl-decahydroisoquinoline was obtained. Treatment with sodium ethoxide and ethyl nitrite gave by ring opening N-acetyl-10-oximinodihydrohomomeroquinene ethyl ester. Hydrogenation of the oximino ester gave the corresponding amine. The latter, in turn, yielded N-acetyl-10-trimethylammonium-dihydrohomomeroquinene ethyl ester iodide with excess methyl iodide in ethanol over potassium carbonate. With concentrated alkalis the quaternary salt underwent normal Hoffman elimination to give the desired vinyl derivative, homomeroquinene, which was isolated as the N-uramido derivative by treatment with excess potassium cyanate

in neutral aqueous solution. Subsequently, the free base was regenerated and converted to N-benzoylhomomeroquinene ethyl ester for ultimate condensation with ethyl quinate (*vide supra*). Over-all yields were small, and the process has little to recommend it for commercial production. This fact does not detract, however, from the credit due these authors for their ingenious solution of an old and vexing problem of organic chemistry.

Woodward, Wendler & Brutschy (24) greatly improved the conventional Oppenauer oxidation of quinine and other Cinchona alkaloids to the corresponding quinones by substitution of alkali alkoxides for the more acidic aluminum alkoxide catalysts. Similar improvement in the related Meerwein-Ponndorf reduction of alkaloidal ketones (e.g., quinone) to the corresponding alcohols was effected. The predominance of reduction products bearing the quinidine configuration in the latter process led to the suggestion of a quinidine-type configuration [cf. Prelog & Zlátný (22)] for quinone and related ketones obtained from the Cinchona alkaloids. Doering, Cortes & Knox (25) achieved one-step partial racemization of quinine by combining an oxidation-reduction system such as fluorenone-fluorenone with a sodium *t*-butoxide catalyst in toluene solution. Sodium isopropoxide catalyst in the Meerwein-Ponndorf reduction of quinone yielded predominantly quinidine, while sodium pentylate-3 directed the reduction principally back to quinine. Alkali *t*-alkoxides gave maximum yields of quinone in the oxidation of quinine by preventing subsequent reduction in the presence of a primary or secondary alcohol. It is of some interest to note that the *epi*-bases contained in quinidine could be converted in good yield into quinone by oxidation with fluorenone in the presence of potassium butylate with benzene solvent.

Methods of separation and analysis.—Ion-exchange reactions with solid reagents such as the sulfonated coals and synthetic resins appear to represent a practically unexploited field for research in Cinchona alkaloid separation and identification. Applezweig (26) adsorbed crystalline components of totaquine on a sulfonated coal and eluted with ammonia in ethanol. Mindler, Sussman & Wood (27) described the general application of this type of adsorbent to alkaloid purification especially in connection with alkaloids such as quinine which are relatively insoluble in water. There is great need for the application of ion-exchange reactions as well as of the counter-current distribution method of Craig *et al.* (28) to the

problem of separating and identifying the components of the so-called amorphous Cinchona alkaloids and of the alkaloids of green Cinchona barks and leaves.

Kyker & Lewis (29) developed a nephelometric method for the determination of quinine in blood and other animal tissues that is based upon the formation of stable suspensions with silicotungstic acid. It appears that the sensitivity of this method can be somewhat increased by the use of lower and more carefully controlled cooling temperatures than those recommended by the authors. For use with similar biological materials Marshall & Rogers (30) and Brodie *et al.* (31, 32, 33) developed a variety of methods including the formation of extractable salts between the Cinchona alkaloids and such acid dyes as bromthymol blue and methyl orange.

For use with all plant tissues the old Dutch Kina Bureau method is still the preferred method of assay. Numerous modifications of this procedure have been described, the most important of which (21) were designed to eliminate interference from the amorphous alkaloidal glycosides of green barks, leaves, flowers, and fruits of Cinchona. These impurities greatly interfere with the crystallization of quinine and cinchonidine tartrates and also with the subsequent determination of the optical rotation of these tartrates.

Stimson & Reuter (34) attempted without conspicuous success to devise a procedure for the estimation of methoxyl-containing Cinchona alkaloids by absorption spectrophotometry at different pH values. Loustalot & Pagan (35) employed a photoelectric colorimeter with regional filters to determine quinine in Cinchona bark extracts. A number of factors inherent in the design of photometers and in the composition of bark extracts are responsible for serious limitations of sensitivity. It is also worth noting that the procedure is incapable of distinguishing between quinine, quinidine, and their corresponding *epi*- and dihydro homologues. In general, methods for direct estimation of Cinchona alkaloids in mixtures that are based upon absorption spectrophotometry or modifications thereof do not appear to hold promise at the present time. To a much smaller extent similar limitations attend the estimation of quinine and quinidine in plant tissue extracts by fluorophotometric procedures. Although a number of laboratories have devised fluorometric methods, few of these have been published so far as the author is aware (36, 37).

Biological production.—Experimentation on the biological production of quinine has long centered in the Netherlands Indies. Much of this information has only recently become available, however, and opportunity is taken herein to summarize important advances which, although not recent, nevertheless will be largely new to workers in the western hemisphere.

Intrinsic factors governing the nature and quantities of alkaloids produced by the Cinchona tree are not well understood. Extensive experience in Java with grafts of high-quinine-yielding clones of *Cinchona ledgeriana* upon rootstocks of the low-quinine-yielding *C. succirubra* led to the conclusion that scion and stock are virtually without specific effect upon one another insofar as quinine production is concerned (38, 39, 40). This suggests that either the loci of quinine synthesis and accumulation within the tree are identical or the relative capacities of stock and scion for alkaloid accumulation have not been altered by grafting. The possibility of occurrence of localized alkaloid synthesis within the Cinchona tree has therefore neither been eliminated nor confirmed. In this connection the report of alkaloid synthesis in detached leaves of *C. succirubra* by Weevers & Van Oort (41) probably has little bearing, since, as Spruit has pointed out (42), the "alkaloid" in this case was undoubtedly the alkaloidal glycoside of unknown structure which Kerbosch & Van Dorssen had isolated from Cinchona leaves (21).

Likewise, there is little information with which to relate quinine synthesis to other vital processes in the living tree. Spruit (43) demonstrated that the bulk of the quinine in the bark of mature trees occurs in the outer layers where the cells are dead or dying. As a result the trunk is continually cutting off its most rich alkaloid deposits from direct contact with the metabolically active tissues of the underlying layers. This fact, coupled with the observation of Kerbosch [cited by Spruit (43)] that the amount of quinine in a unit surface area of trunk bark always increases and never decreases during growth, suggested to Spruit that quinine (and, by inference, also its associated alkaloids) is a by-product of metabolism.

Heredity as an intrinsic factor governing alkaloid production appears from the Dutch literature and from the more recent work of Martin & Gandara (11) to exert both qualitative and quantitative types of controls. Kerbosch & Spruit (44, 46, 49) and Spruit (45) conducted a long-term program for the clonal evaluation of

Javan Cinchonae. Relative productivity was judged by a combination of individual criteria including (a) the dry weight of one square decimeter of trunk bark, (b) the quinine content of this bark, and (c) the circumference of the trunk—all at one meter from the ground. The height of the trunk was also determined. Ratings of clones under test were usually based upon the productivity of a standard clone under field conditions. The quinine yield of any given tree or group of trees belonging to one clone was given by an equation of the type $Q = pcw(h-10)/3$ where p is percentage of quinine in the bark sample, w is the weight of the sample in grams per square decimeter, c is trunk circumference and h is height of the trunk in decimeters. To obtain an expression for quinine production per unit of planting area, this equation was modified by inclusion of an additional factor representing the number of trees on such an area. Relative productivity of different clones was usually expressed in terms of percentage of the yields of the standard clone based upon the simple equation, $QPR = pcw$, where the terms are the same as above. QPR represents the amount of quinine in a ring of bark one decimeter wide and extending around the trunk at a distance of one meter from the ground. In certain cases, the ratio between the amount of quinine per ring and the amount per tree was represented by a relatively constant ratio (44 to 48). In practice, however, the irregularities in growth that were introduced by a progressively impoverished soil had to be eliminated by using the equation in the following form $Q_r = pcw/(h-10)^2$. With such indices of productivity large differences between the inherent producing capacities of various clones derived from the original Ledger mother trees were demonstrated. Indeed, some these outyielded the original mother trees by considerable margins (49).

Extrinsic (i.e., environmental) factors eventually introduced further conflicting trends, however, as the soils upon which the tests were made became more and more infertile. Kerbosch & Spruit (50) early suspected the nature of the difficulty, and Spruit (45) finally announced that it was due to fundamental differences in response of various clones to different levels of soil fertility. Thus, some clones increased their yields only slightly with increase in soil fertility, while still others increased them more rapidly. This illustrates an interesting type of adaptation to environment so far as alkaloid production is concerned. From the point of

view of the chemical economy of the organism its significance is not yet clear.

On the theory that the actual yield of a given planting of trees can be expressed as a relatively simple function of a hypothetical maximum yield, viz. $A = A_m a_1 a_2 a_3 \dots a_n$ (where a_1, a_2 , etc., are values lying between 0 and 1 for factors which influence yield), Kerbosch & Spruit (49 to 53) attempted to ascertain the effect of environmental variables upon quinine yield. Phosphorus and particularly nitrogen were shown to be the principal limiting factors in Javan soils. Consequently, regular fertilizer programs were adopted for Cinchona plantations. Independent estimates of the fertilizer requirements of the crop were obtained by correlating yields with the results of soil analyses and crop analyses on the one hand and with calculations obtained with the Mitscherlich equation, $\log (A - y) = \log A - cb$, on the other. In this case A is the maximum possible harvest, y the actual harvest, b the quantity of the limiting element that is available, and c is a factor which is thought to be constant for a given element. For the elements nitrogen, potassium and phosphorus the two methods of estimating fertilizer requirements showed very good agreement with the result that quinine production was placed upon a near-quantitative basis with respect to these variables. The same investigators in an extensive study of the Cinchona soils of Java identified other major factors that control quinine production, e.g., the amounts of air, sand, humus, and calcium in these soils, the water retention of the soils, and the degree of base-exchange capacity and of base saturation of the soil-clay fraction (54, 55). These findings have also been discussed at length in the recent book on the soils of equatorial regions [Mohr (56)].

In retrospect, the researches of the Cinchona Experiment Station in Java constitute a brilliant chapter in the much-neglected field of biological engineering, and it is thus all the more ironical to note that the very excellence of this work led to serious economic difficulties in the quinine industry prior to the last war partly as a consequence of overproduction (57).

Recent trends in Cinchona research appear to have dealt chiefly with such problems as (a) a broadened base for clonal selection involving such features as growth vigor, bark thickness, sensitivity to soil fertility level, disease resistance and root vigor; (b) recombination of these features in improved varieties by con-

ventional plant breeding methods; (c) development of root vigor in Ledger clones sufficient to remove the necessity for grafting (46, 49); and production of antimalarials from *C. succirubra* grown as a short term (two-year) crop in Russia and in the western hemisphere (58, 59). In this last connection, attention should again be called to the early work of Van Leersum (18), Tatarskaja & Solomko (20), and Kerbosch & Van Dorssen (21).

THE NICOTIANA ALKALOIDS

Of the eleven known tobacco alkaloids, Ernst Späth has isolated, adduced structural evidence, and/or synthesized each. Equally important have been his contributions to the knowledge of molecular structure in other alkaloidal groups. The accomplishments of his students have been many; among them may be mentioned the facile synthesis of physostigmine by Julian & Píkl (60). It is with a distinct feeling of loss, therefore, that his passing is recorded in this seventh of a series of reviews to which he was an early contributor (61).

New records of occurrence.—The widespread occurrence of nicotine in the plant kingdom is a matter of singular interest (62). Pal & Narasimham (63) have added still another species to the list, namely *Eclipta alba*, a member of the Compositae.

Markwood demonstrated that certain flue-cured tobaccos contain important amounts of nornicotine (64). In view of the lower volatility of nornicotine, as compared with nicotine, this observation may help to explain the milder smoking properties of these cigarette tobaccos but also casts doubt upon the accuracy of virtually all previously reported determinations of "nicotine" in tobacco tissues (*vide infra*).

Shmuck & Borozdina (65) and Smith & Smith (66) have investigated the alkaloids of a large number of wild *Nicotiana* species. Nicotine, nornicotine, or anabasine was the major alkaloid in each case. No evidence was found for the presence of appreciable amounts of the other known tobacco alkaloids listed by Späth & Kesztlér (67) although the possibility of their presence is not excluded.

Dawson (68, 69) showed that, in addition to the principal alkaloid anabasine, either nornicotine or nicotine is a characteristic constituent of *Nicotiana glauca*, depending upon the particular strain or variety examined. Kostoff (70) and Smith & Smith (66) have summarized the literature regarding the interesting hybrid,

Nicotiana tabacum \times *N. glauca*. Dawson has shown (69) that the so-called anabesine of these authors is actually a mixture of nornicotine and anabesine and that nornicotine may be present in very substantial proportions. This would appear to remove the hybrid from the list of potential commercial sources of anabesine for insecticidal use (71).

The steam-volatile alkaloids of various species of *Duboisia* have been examined by Bowen (72), by Hills, Trautner & Rodwell (73), and by Bottomley, Nottle & White (74). It appears probable that nicotine and/or nornicotine will be found to occur in most of these species.

New syntheses.—Notwithstanding the fact that supplies of insecticidal nicotine continue short, there appears to be little activity directed toward the economical synthesis of this alkaloid. Woodward, Eisner & Haines (75) obtained myosmine in about 18 per cent yield by pyrolysis of nicotine over quartz. This alkaloid had earlier been obtained from cigar smoke by Späth, Wenusch & Zajic (76). Haines, Eisner & Woodward (77) obtained *dl*-nornicotine in 93 per cent yield from myosmine by reduction with hydrogen and palladous oxide at room temperature. A comparatively cheap and abundant source of nornicotine has thus been made available for experimental purposes. In connection with the earlier synthesis of myosmine by Späth & Mamoli (78), the equilibrium that is set up between myosmine and 3-pyridyl- ω -aminopropyl ketone in water (77) is of some interest. As a consequence of this equilibrium, myosmine in water yields the theoretical amount of primary amino nitrogen with nitrous acid in the Van Slyke machine. Likewise, the corresponding oxime and phenylhydrazone may be prepared (77).

Frank, Holley & Wikholm (79) prepared nicotyrine by catalytic dehydrogenation of nicotine with palladium on asbestos. Späth & Bodenberger (80) racemized *l*-nicotine by heating the dimethiodide.

Properties and methods of separation.—Effective procedures for the purification and positive identification of nornicotine from plant sources have until recently been difficult to devise. Perhaps the most important single contribution to the solution of this dilemma has been the azeotropic removal of nicotine with water at the boiling temperature as described by Smith (81) and by Kelley, O'Connor & Reilly (82). Neither nornicotine nor anabesine form such azeotropes. Since anabesine is not ordinarily present in de-

tectable amounts in commercial varieties of *Nicotiana tabacum*, this means that a quantitative separation of nornicotine from its companion alkaloid nicotine is readily achieved. Formerly, reliance was placed upon fractional distillation or more usually upon fractional crystallization of the mixed picrates. Bowen & Barthel (83) have shown that the picrates of nornicotine and nicotine form eutectics and eutectic mixtures when coprecipitated. Dawson (69) observed that nornicotine and anabasine picrates also form a eutectic. It is thus clear why, in both cases, great difficulty has attended efforts at separation by fractional crystallization (66, 70, 84).

Picric acid still remains the most practical reagent for use in the preparation of easily handled and readily purified derivatives of the tobacco alkaloids. Nicotine and anabasine dipicrates are nicely crystalline and may be recrystallized easily from hot water or alcohol. Nornicotine dipicrate, on the other hand, usually separates from water or alcohol as a yellow oily turbidity or as a reddish gum. Crystallization occurs on standing and can be hastened by scratching provided the amount of impurity is not high. Unfortunately, however, nornicotine dipicrate has a tendency to crystallize with small amounts of various impurities and with excess picric acid. Hence, repeated recrystallization is often necessary. Crystals of nornicotine dipicrate are usually hard and stubby and when pure possess a relatively clear-cut melting point free from decomposition. It is an interesting fact that, when heated in water, nornicotine dipicrate reverses its behavior on cooling in aqueous solution and exhibits a yellowish turbidity before dissolving. This may be used in routine work as presumptive evidence for the presence of nornicotine.

The eutectic point of the system nicotine-nornicotine-picric acid, according to Bowen & Barthel (83), probably occurs in the vicinity of thirty parts of nicotine to seventy of nornicotine. The eutectic point of the anabasine-nornicotine mixture corresponds to approximately ten parts of anabasine dipicrate to ninety parts of nornicotine dipicrate. Dawson (69) found that excess separation of nornicotine dipicrate, as a second liquid phase before crystallization is induced, may strongly alter the melting point and solubility of the resulting mixed picrates of nornicotine and anabasine.

When nornicotine is accompanied by anabasine in the residues from the azeotropic distillation of nicotine by the method of Smith (81), it may be separated for purposes of qualitative identification by methylation to nicotine, using formic acid and formaldehyde

according to the method of Shmuck (85) and Markwood (86). Under the conditions employed in the latter procedure appreciable methylation of anabasine does not occur. The methylated nornicotine may be separated readily from anabasine by fractional crystallization of the picrates.

Analytical methods.—Prior to Markwood's discovery of nornicotine in certain strains of cigarette tobacco, the results of alkaloid analyses on tobacco tissues were referred to "nicotine." It has since become established that nornicotine occurs in variable proportions in a wide variety of commercial tobacco strains (87). Hence, virtually all published quantitative data on the nicotine content of tobacco tissues are suspect unless and until nornicotine can be shown not to occur in such tissues under the usual range of cultural conditions. It may be somewhat more accurate to refer these data to "total alkaloid" rather than to nicotine, although this is usually permissible only when the alkaloids have been extracted from the alkalinized tissues by immiscible solvents. Even in this case there may occur an error which arises from differences in molecular weights of nicotine, nornicotine and the accompanying minor alkaloids. Analytical data that have been obtained by steam distillation may have large additional errors due to the lower volatility of nornicotine, unless the distillation has been exhaustive.

Perhaps the most accurate and sensitive method to be devised for the assay of nicotine and nornicotine in plant tissues is the colorimetric determination of the pigments produced by the action of cyanogen bromide and a coupling agent upon these alkaloids. Among the more recent modifications of this method may be mentioned those of Larson & Haag (88) and of McCormick & Smith (89).

Dawson (69) devised a proximate method for the determination of nornicotine in the presence of anabasine following removal of nicotine by azeotropic distillation. The melting points and solubilities of the purified mixed picrates were determined and compared with standard curves obtained with mixtures of known composition. In view of the differential solubilities of the picrates of these two alkaloids in water, the limitations of such a method are obvious.

Shmuck (85), Bowen & Barthel (90), and Markwood (86) have converted nornicotine and nornicotine dipicrate into nicotine by methylation with formaldehyde and formic acid. Markwood (86)

converted the nornicotine component of mixtures into N-nitroso-nornicotine with nitrous acid. Nicotine was distilled with steam and determined directly. In an aliquant portion nornicotine was quantitatively methylated and the whole distilled again as nicotine. Nornicotine was calculated by difference. Bowen & Barthel (91) preferred to eliminate the methylation step and to distill the mixture of alkaloids directly from concentrated sodium hydroxide-sodium chloride solution. These conditions seem drastic for so labile a substance as nicotine. There is some reason to believe that the stability of N-nitrosornicotine during steam distillation is not so great as was formerly thought. Thus, there is still need for a good chemical method for the quantitative separation of these two alkaloids.

Trautner & Neufeld (92) developed an interesting variation of the usual titrimetric procedure by carrying out the neutralization of nicotine in a chloroform solvent with picric acid as reagent. Smith (81) and Smith & Smith (66) have determined nicotine and nornicotine by azeotropic distillation of the former and titration of the distillate.

Biological production.—Considerable progress has been made in the investigation of alkaloid production among the *Nicotianae*. Dawson (93) has elsewhere reviewed the developments that led to discovery of the localization of nicotine synthesis in the root system and of nornicotine synthesis in the shoot of the different species of *Nicotiana*. Nornicotine was shown to represent a secondary conversion product of nicotine (69, 71). Anabasine synthesis was found to occur in all organs of the plant body (68, 69). The mechanics of alkaloid distribution within the tobacco plant are therefore beginning to be understood, but the mechanisms of synthesis remain to be elucidated.

Kostoff (70) has summarized the literature that purports to demonstrate the genetical dominance of anabasine synthesis over nicotine synthesis in grafts or hybrids containing the two systems. Dawson (69) has shown this view to rest on the presence in the grafts and the inheritance in the hybrids of the normal system for nicotine demethylation and the confusion of the resulting nornicotine with anabasine. It may now be stated categorically that there exists no evidence for a relationship between the synthetic mechanisms or the inheritance of any of these alkaloids with the single exception of the necessity for a supply of nicotine in the shoots of plants which have inherited the nicotine demethylating

mechanism before the presence of this mechanism can become manifest.

The quantitative aspects of tobacco alkaloid inheritance have received attention from a number of quarters. Koenig (94) and Valleau (95) have selected strains that contain exceedingly low contents of nicotine and of total alkaloids. Similarly, *Nicotiana rustica* has been bred and selected for exceptionally high yields of nicotine (96, 97). Although a number of attempts have been made to evaluate polyploid *Nicotianae* (i.e. plants containing whole-number multiples of the basic chromosome number), the lack of a legitimate basis for comparison has seriously limited their value (66, 70, 98).

Extrinsic factors that govern alkaloid production in commercial tobaccos have been examined by McMurtrey, Bacon & Ready (99). Favorable conditions for maximum yields appear to be highly fertile soils, relatively heavy soil texture, adequate and well distributed water supply⁴, and early removal of tops and suckers. It appears that a mathematical formulation of the effects of these factors upon relative growth rates of root and shoot and thence upon nicotine production rates might be made in the light of present knowledge of the respective roles of root and shoot in nicotine synthesis and accumulation [cf. Berthold's (100) qualitative observations on the root/shoot ratio of tobacco as modified by environmental variables].

The data presented by Garner *et al.* (101) can be recalculated to show that progressive increments in available soil nitrogen influence leaf dry weight yield and nicotine production more or less independently. High rates of nicotine output appear to result from luxury nitrogen nutrition. The validity of this conclusion cannot be assured, however, until concomitant effects upon root dry weight production have been obtained.

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⁴ This observation applies to *Nicotiana rustica*. It appears not to be universally true for other types and crops of tobacco, however.

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PHOTOSYNTHESIS

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INTRODUCTORY REMARKS

Concluding the previous review (1), French stated that

until the last few years photosynthesis has been considered as a relatively simple system of a few closely co-ordinated reactions which might be analyzed kinetically by rate measurements under different conditions. . . . It now seems that this approach, if only applied to the over-all reaction, may be inadequate to lead to a complete description of the process of photosynthesis. The tendency at present is to attempt to study one major step at a time.

Certainly, these considerations are true in part; we only need to recollect that, in 1918, Willstätter & Stoll (2) attempted to demonstrate complete photosynthesis *in vitro* with pure chlorophyll, even though the complexity of the process had long been recognized; it had led Blackman (3) to the introduction of his extremely fruitful principle of "limiting factors."¹ The application of this concept by Warburg (4) initiated further useful lines of analysis not compiled here. Moreover, the known complexity of respiration may have contributed to suspicion of considerable complexity in photosynthesis. Thus, the reviewer believes that advances made in recent years are not wholly the result of a change in mental attitude, but are in part achieved by new methods of approach.

New lines of investigation started to some extent from very old stocks. A few examples may be mentioned. The study of the dark fixation of carbon dioxide (2) was carried further, using tracer isotopes (6). The study of chlorophyll fluorescence (7, 8), preferably including measurements on gas exchange, was extended to new types of organisms, e.g., purple bacteria (10) and diatoms (11) and was applied to special subjects, e.g., to studies on the so-called induction period (11), and to the role of accessory pigments (12, 13). Photochemical oxygen evolution was the first important phenomenon in photosynthesis accessible to studies *in vitro* (14, 15), in which the operation of hydrogen-acceptors others than carbon dioxide is especially interesting. Water is generally accepted

¹ We have to regret Blackman's recent death (January 30, 1947, aged 80) (5).

as the ultimate reductant (16, 17, 18). In the period covered by this review, consideration of redox potentials in connection with photosynthesis (19, 20) was taken up anew (18, 21, 22, 23). No appreciable experimental development seems to have been achieved concerning the possible role of phosphate compounds as energy carriers in photosynthesis. In the following, a few "main lines" will be discussed in some detail; from numerous more incidental observations a number of those yielding valuable contributions to a completion of the picture, or representing special fields, will be briefly considered.

QUANTUM EFFICIENCY AND RELATED QUESTIONS

It might appear that much more work and discussion has been devoted to quantum efficiency studies than is in accordance with their importance for the ultimate understanding of the course of photosynthesis. As soon as it became obvious that Warburg's original yield of 1:4 (0.25) was not obtainable under all experimental conditions, the theoretical importance of yield determinations was no doubt decreased. If the number of 4 quanta suggested a simple mechanism for reduction of carbon dioxide to $[\text{CH}_2\text{O}]$ requiring the introduction of two hydrogen and the removal of one oxygen, the higher numbers forced the acceptance either of considerable energy losses, or of a mechanism requiring a large number of photochemical steps, the course of which could not be traced. The divergent results from various laboratories stimulated investigators to attempt a definite answer, with at least fault-free technique. Previous reviewers (24) stated:

... it is not important whether the maximum yield is 0.08 or 0.12. But it is of crucial importance whether the yield is high (~ 0.25) or low (0.08–0.11).

Rieke (25), following Warburg's original procedure, had confirmed his results, and Emerson & Lewis (26) had shown that Warburg's supposition that $\text{CO}_2:\text{O}_2$ was close to unity was not valid for his yield determinations, even better "quantum yields" than 0.25 being obtainable. American investigators gradually have agreed at 0.12 to 0.08 as the yield under normal conditions [cf. (21)]. Tonnelat (27), studying *Chlorella pyrenoidosa* in thick suspensions in a calorimeter, in green light, obtained an efficiency of

30% [of the thermodynamical energy requirement] ce qui correspond à l'absorption de 8 quanta de lumière verte par molécule réduite.

Thus matters might appear relatively settled if, recently, some new stimulatory points had not been raised. Warburg (28) found the quotient $\text{CO}_2:\text{O}_2$ to be 0.95 under conditions similar to those applied for the yield determinations in 1923. He concluded that the efficiency remains fixed at 0.25 and that his result invalidated the carbon dioxide-outburst at the start of illumination.

Wassink *et al.*, in incidental observations, found quantum numbers of 0.11 to 0.2 in thin suspensions of *Chlorella* under prolonged illumination (9, 29). These numbers appearing somewhat more favorable than those found by American authors, a more detailed study was undertaken by Kok (30). The quantum yield of *Chlorella* was determined in thin suspensions using simple Warburg manometers and conical vessels with clear walls, in experiments of prolonged duration in sodium light. Light absorption in the suspensions as used was estimated by the white sphere method (31). Kok studied this method fundamentally and improved the technique of measurement. Quantum yields were determined from the slope of a graph through points obtained at various light intensities. Neutral or slightly acid media gave better yields than the alkaline Warburg buffers. [For this point cf. also Myers (32), Rieke (25), and Emerson & Lewis (33).] As the result of a statistical analysis of numerous observations Kok arrived at a yield of 0.13 for nonbuffered media with carbon dioxide-supply, and of 0.103 for Warburg buffer No. 9. His technique of cultivation still seems susceptible to some further improvement, but the above values may well be regarded as representative for cell-populations.

It should be kept in mind that, so far, we have no knowledge regarding the theoretically more interesting optimal yield for a single cell, and attention may be drawn to Linderstrøm-Lang's techniques, especially in their development by Zeuthen (34). Kok supposes 0.17 to be the theoretical yield at light intensities well above the compensation point.

Kok's most interesting observation seems to be that the curve for the rate of photosynthesis versus light intensity invariably cuts the ordinate above the origin after correction for dark respiration. This means that respiration is reduced during illumination, and Kok found the reduction to be 50 per cent within narrow limits. This implies that at low light intensities the quantum efficiency may be twice that at higher intensities. So far as is known, the ratio $\text{CO}_2:\text{O}_2$ is the same. Kok's interpretation is that respiration

is completely suppressed in light, and replaced by a process showing no gas exchange, and converting light energy with high efficiency into a form of energy available for cell-metabolism. The suppression of the uptake of a molecule of oxygen in respiration would require less quanta than the evolution of a molecule of oxygen in photosynthesis, thus suggesting photosynthesis with a high yield below the compensation point. Kok's findings may possibly reconcile high efficiencies recorded below the compensation point, and low ones beyond this point, so that, dependent on the average light intensity in the suspension, values of 0.25 to 0.10 might be found.

Much will depend upon the generality of the occurrence of the phenomenon observed by Kok. Some observations seem to favor unchanged respiration during photosynthesis, e.g., those in which, at various temperatures with light a limiting factor, equal net assimilation rates are found, after correction of widely different oxygen production with widely different rates of (dark) respiration (11). Something similar has been observed in connection with cyanide inhibition (35).

In view of Emerson & Lewis' results (26) it is not clear why Warburg (28) did not find appreciable deviations from unity of the photosynthetic quotient. Wassink & Kersten, in a single experiment with *Chlorella*, found about the same value for the photosynthetic quotient in accumulated 10 sec. illuminations [technique cf. (11)] and under continuous illumination. The ratios $O_2:CO_2$ were 1.14, 1.18, 1.12 respectively for intermittent, and 1.17, 1.18, 1.16 for continuous illumination at three different light intensities (unpublished).

With diatoms from a fairly unialgal natural accumulation, Wassink & Kersten (11) in some incidental estimations of the quantum yield found about 0.08 for dilute suspensions in Warburg buffer. This agrees well with Dutton & Manning's findings (36), viz. 0.06 to 0.10 by a technique also measuring only oxygen exchange.

Quantum efficiency studies in purple bacteria are complicated by the S-shaped curve of photosynthesis against light intensity. The values in reference (10) referred mainly to the steepest part of the slope. Deviations in general did not exceed 10 per cent [cf. (37)]. Manten (38), studying phototaxis of *Rhodospirillum rubrum*, gave indirect support to the "donor" theory of the S-shape (10).

The spectral sensitivity distribution and the dependence of the contrast sensitivity on the incident intensity suggests that phototaxis is chemotaxis upon products of photosynthesis in this organism. At low light intensities the phototactic contrast sensitivity was constant, indicating the same for the quantum yield of photosynthesis. Manton's experimental conditions were not especially suited to a study of the relation between internal and external supply of compounds involved in photosynthesis, since he observed phototaxis in the peptone culture medium. Schrammeck (39) had indeed found a decrease in contrast sensitivity, but only at very low light intensities (between about 0.05 and 0.005 MK). This decrease may be connected with quantum-statistical phenomena, and Wohl's (40, 41) considerations may hold here. Staverman considers the S-shape as a whole due to "quanta-collecting" reactions (41a).

French (1) suggested that determination of the carbon dioxide-donor relation at low light intensities might enable a check on the "donor competition theory." However, if internal conversion with uptake of light energy involved only very small gas exchanges, no appreciable change in the measured carbon dioxide-donor relation would be found.

Another point of very considerable theoretical interest is raised in the already reviewed (1) efficiency studies of Emerson & Lewis (42), showing that light of wave lengths longer than those corresponding with the fluorescence maximum of chlorophyll is of reduced photosynthetic activity. This would mean that the absorption curve does not fully evaluate potential excitation to photosynthesis. It would mean, moreover, that photosynthesis, and probably fluorescence, do not result from the lowest excitation-level in the chlorophyll molecule, and that near the "photosynthetic" level a lower level exists with a low transition probability between them. Incident light of the wave lengths under discussion would then be expected to give deviations in fluorescence yield and fluorescence spectrum. This would mean considerable emendation of current views, and one would prefer a more simple explanation.

FLUORESCENCE AND THE ROLE OF PIGMENTS IN PHOTOSYNTHESIS

Wassink & Kersten (11) made a combined study of photosynthesis and chlorophyll fluorescence in diatoms, using nearly unialgal natural material [*Nitzschia dissipata* (Kütz.) Grun.], or unialgal

cultures derived from this material. Some purely photosynthetic data may be of interest, too, since quantitative studies of diatom photosynthesis are scarce. The "Blackman" capacity was large, enabling utilization of high incident energies at low temperatures, which may explain the abundance of diatoms early in spring. The photosynthetic quotient $O_2:CO_2$ was about 1.1, in accordance with earlier observations of Barker (43); the same value was found for *Chlorella* in simultaneous experiments. This suggests that fat formation does not take place directly in photosynthesis [cf. also Barker (43), Harder & von Witsch (44) and von Witsch (45)]. Cyanide and ethylurethane had much the same effect as in *Chlorella*.

The fluorescence yield was about 0.25 per cent. The curve for fluorescence *vs.* incident intensity showed a decrease in yield beginning at an intensity about that for light-saturation of photosynthesis. This decrease was absent under carbon dioxide-limitation. It was suggested that the limited capacity of the oxygen-producing (OH-removing) system is normally responsible for light saturation. The lack of sufficiently quick removal of OH-groups would render the transfer system more oxidized, and, in conjunction with earlier observations (46), would lower the fluorescence yield. Thus, in this case, decrease of fluorescence yield accompanied decrease of photosynthetic yield, a warning against a too ready assumption of "antiparallelism" between photosynthesis and fluorescence!

Correlated observations on photosynthesis and fluorescence were also made in illuminations of a few seconds, starting from the previous observation (46) that in the initial phase, a certain fluorescence yield—and thus, probably, a definite state of the cell—can be maintained by alteration of light and dark periods of sufficient duration. Most observations were concerned with light of five or ten seconds duration per minute. At low light intensities the rate of photosynthesis was similar to that under continuous illumination, but the light saturation level was much lower.

This level was temperature-dependent, but, at least in *Chlorella*, insensitive to cyanide (unpublished). Fluorescence in relation to light intensity behaved as under carbon dioxide-restriction, the yield decrease at light saturation (cf. above) not being observed. The general conclusion was that in darkness a dark chemical, cyanide-sensitive system is partly inactivated. In the light it is quickly restored as, e.g., a comparison of the light saturation level

in 5 sec. and 10 sec. experiments showed. The restoration probably means a shift to the oxidized side. The fluorescence results suggest that the dark chemical system under consideration belongs to the "carbon dioxide side" of the photosynthetic chain.

Fucoxanthin was found active in photosynthesis with a yield comparable with chlorophyll (12). Combined measurements of the yield of photosynthesis, and of chlorophyll fluorescence, in spectral regions involving only absorption by chlorophyll or absorption by chlorophyll and fucoxanthin proved that the energy absorbed by fucoxanthin is used only after transfer to chlorophyll. These investigations were carried out during the war; independently, very similar studies were carried out by Dutton & Manning (36), and by Dutton, Manning & Duggar (13), with similar results. Curiously, also the "rediscovery" of chlorophyll-*c* was reported from both sides (12, 47). Dutton *et al.* additionally studied the effects of high light intensities at various wave lengths; Wassink & Kersten payed additional attention to the spectral properties of the pigments in the living cell, in cell-free colloidal extracts, and in solution. Wassink (48) discussed the importance of fluorescence spectra studies for problems of energy transfer in chloroplasts. Förster's critical study on energy transport and fluorescence (49) brings out that "immaterial" energy transfer without quenching over as much as 1000 dye molecules is conceivable provided association is impossible. For chlorophyll the required conditions might be realized in the chloroplasts. As far as we know, Franck's proposed critical comparison of the results of his group and ours on photosynthesis and fluorescence [cf. (1), p. 408] has not yet appeared.

Manten (38) confirmed on a quantitative basis the older observations on carotenoid-sensitized phototaxis in *Rhodospirillum rubrum*. However, the chief carotenoid, rhodoviolascin (spirilloxanthin) was inactive. Evidence was strongly in favour of the view that photosynthesis is at the base of the phototactic stimulus. This would mean that some of the carotenoids are active in photosynthesis. This result is not necessarily in conflict with French's earlier findings (50) of the general concordance of the photosynthetic action spectrum with the absorption spectrum of bacterio-chlorophyll, since the participation of a small carotenoid fraction in photosynthesis in a suspension of bacteria would be very difficult to establish.

Making use of the established stabilizing effect of hydrogen

sulphide on bacterio-chlorophyll in solution (31), Manten obtained this pigment in a probably pure state, by chromatography. The absorption spectrum is of the type of that of chlorophyll-*a*, but "wider," with maxima at about 3700 Å and 7700 Å in benzene. By chromatography, too, Manten obtained a series of red and orange carotenoids from extracts of *Rhodospirillum rubrum* (38). Wassink & Lubberink (51) found a very similar mixture in *Chromatium*, strain D, which, moreover, showed a close resemblance to Karrer & Solmssen's findings with *Rhodovibrio* (52). Additionally, a pale-yellow carotenoid was found, accompanying bacterio-chlorophyll in the primary extraction with dilute ethanol, and, therefore, being probably of xanthophyllous nature. Van Niel studying the colour change of the Athiorhodacea *Rhodopseudomonas spheroides* upon aeration (53) found chiefly one red carotenoid and one yellow one, the yellow one probably being converted into the red one upon aeration of living bacteria. The pigments were related to some found by Karrer *et al.*

Haxo & Blinks (54) presented strong evidence for phycobilin photosynthesis in red algae, thus complementing the experiments of Emerson & Lewis (55) with blue-green algae. They used a modified polarographic method to measure oxygen production. Species with differently colored bilin complexes showed different spectral activity maxima.

For many spectro-biological studies

there is a need for a much greater total amount of energy than is obtainable from ordinary commercial monochromators, and for greater spectral purity than is possible from filters (56).

This fundamental technical problem was solved independently by various American investigators, e.g., by Emerson & Lewis (42), and by French, Rabideau & Holt (56), who constructed large grating monochromators, and by Parker *et al.* (57), who built a prism-monochromator.

Using their apparatus in conjunction with an Ulbricht sphere Rabideau, French & Holt (58) determined absorption spectra of leaves, of suspensions of chloroplasts, and of their supersonic extracts. They noticed the smaller depth of the absorption minimum in the green in leaves as compared with the less diffusing systems, and correctly connected with this the differences in the length of the light path. Kok (30) made model studies to obtain further insight into interrelations between scattering and absorption.

Chlorophyll may be active as light-absorbing pigment in photo-periodic influences on floral initiation (57). The low energies required and the delayed appearance of the reaction indicate that this process like, e.g., phototropism, but unlike photosynthesis, is of the "stimulus" or "amplifier" type, the light only "directing" another, internal source of energy (59 to 62). The "amplifier" character of phototropism (coupling of a "stimulus" source of energy with an "operating" one) was particularly clear in the blue-green alga *Tolypothrix sp.* Weak, blue light applied on one side induced phototropic curvature only if a sufficient amount of photosynthetically—but nonphototropically—active light was supplied at the same time. Thus, in this case, photosynthesis was the direct source of energy for the phototropic curvature [Manten (38)].

OXYGEN EVOLUTION FROM ISOLATED CHLOROPLAST-SYSTEMS; REDOX POTENTIALS

French *et al.* continued their studies on oxygen evolution of isolated chloroplasts in Hill's system, following the rate of the reaction by constant pH-titration. Photooxidation was reduced in a nitrogen atmosphere and photodecomposition excluded by use of a red filter. Some data on kinetics are presented (16). It is of special interest that supersonic extracts show considerable activity. This may be a first step toward isolation of involved enzymes (63). The list of substances suitable as H-acceptors in the photochemical reactions of chloroplast preparations is extended by, e.g., chromate, sodium meta-vanadate, and by various indicator dyes. The latter become decolorized during the reaction: $2D + 2H_2O \rightarrow 2H_2D + O_2$ (64, 65). Use is made of the circumstance that atmospheric reoxidation of the dye (D) is slow.

Aronoff, like Warburg & Lüttgens (17), used quinoid structures for photochemical oxygen evolution by chloroplast preparations. He reported (18) that the rate of oxygen production in suspensions of granules² is correlated with the redox potential of the quinone. Later (66) he found that at low light intensities the relation was less clear, and even tended to the reversal. At least one dark reaction occurs in addition to the light reaction. Sharply centrifuged preparations of low chlorophyll content showed a remarkably high activity. Intact algae reduced quinone with oxygen evolution.

² It would seem advisable to restrict the term "grana" to structural entities in the chloroplasts.

Chlorophyll in solution was inactive. Since the process still appeared to be complex, Aronoff's conclusion that "the only mechanism of which we may be certain is the fission of water in the 'light reaction'" might not yet be fully warranted. The reviewer has advanced a few facts (21) suggesting that in green plant cells water—like the hydrogen donor in purple bacteria—is involved in a dark process before influencing the energy transfer. It would be important to know whether pigment-protein systems from purple bacteria are capable of reactions similar to that of Warburg-Lüttgens.

Wassink (21, 22, 23) tried to relate photosynthesis and redox potentials in suspensions of *Chromatium*, strain D, from the viewpoint that, essentially, photosynthesis is an oxidation-reduction process. At pH 6.6 upon illumination, the potential shifted towards the oxidized side, strongly in atmospheres of N_2 - CO_2 , less in N_2 - H_2 - CO_2 , still less in N_2 - H_2 . This is exactly what would be expected if the potentials reflect the state of oxido-reduction of some essential link in the chain of photosynthesis. Roelofsen's preliminary result (20) that the potential shift is larger in the presence of a hydrogen donor than in its absence was not confirmed. At a pH of about 8.0, in N_2 - H_2 a potential shift towards the reduced side was often observed upon illumination. This was related with the activation of a hydrogenase by light. Under these conditions, without addition of carbon dioxide, in the light a considerable amount of hydrogen was absorbed by the bacteria. Preliminary observations on the action of cyanide and of hydroxylamine were made. The pigment-protein complex in these bacteria, and perhaps in all photosynthetically active organisms, may be considered as a photo-dehydrogenase.

Boichenko (67, 68), also starting from the concept of photosynthesis as a redox reaction, claims to have demonstrated absorption of carbon dioxide and evolution of oxygen in films of dried chloroplasts, at pH about 8 in the presence of 0.1 per cent fructose which is supposed to act as a hydrogen carrier on the surface of illuminated chloroplasts. Oxygen evolution was estimated by oxidation of leucodyes. Aronoff (66) failed to confirm Boichenko's results by manometric technique. It should be kept in mind that coloration of leucodyes is no strict proof for the production of molecular oxygen [cf., e.g., van Niel's criticism (69) of experiments of Czurda]. Gurevich (70) studied the reduction of *o*-dinitrobenzene by leaves and isolated chloroplasts in the light. In leaves the final

product was *o*-nitraniline; chloroplasts were less active, and formed chiefly *o*-nitrophenylhydroxylamine. *o*-Dinitrobenzene is considered as the acceptor for hydrogen formed from water in a photochemical process.

VARIOUS SUBJECTS

Under this heading a brief and incomplete record will be given of certain studies devoted to other phases of the field, among which are very important lines of attack, for which exigencies of space do not permit full consideration.

Pigment-protein complexes.—The recent development of protein-analysis [cf. (71)] may be fruitful for further study of the protein-bearers of the chlorophylls. During the last years results were reported, e.g., by Stoll & Wiedemann (72), Stoll, Wiedemann & Rügger (73), Timm (74), Smith (75), Comar (76), Fishman & Moyer (77, 78). In a private conversation Professor Stoll mentioned that recently histidine was found in chloroplastin-protein, which brings the results of his group into closer connection with those of other investigators. Timm's analyses were very analogous to previous ones of Chibnall, and showed only small differences between "cytoplasmic" and "chloroplastic" substance. Some data of Timm, in per cent, compared with those of Stoll *et al.* (between brackets) are:

Arginine 14.4 [5.5], histidine 3.6 [0], lysine 4.7 [9.4], cystine 1.6 [1.2], tyrosine 2.6 [3.5], tryptophane 2.1 [2.2], phenylalanine — [4.1].

Du Buy & Woods (79) measured the absorption spectrum of a nucleoprotein solution from purified plastids. Roberts (80, 81) and Southwick (82) concluded from electron microscope studies that several consecutive orders of structures are distinguishable in chloroplasts, chromoplasts, and starch grains. Functional elements of less than 100 Å are assumed, and relations between virus particles and chloroplasts are suggested, similar to those advocated by Woods & DuBuy [e.g. (83, 84)]. However, Algera *et al.* (85) working with le Poole's microscope (86) could not confirm the above observations. The carotenoids of green leaves are always found associated with the chlorophyll (75, 79). Very probably phycocyanin is a separate complex (87, 88). Wassink (48) made a few suggestions for nomenclature.

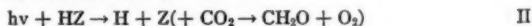
Heterotrophism in photosynthetic organisms.—Purple bacteria show both photo-autotrophic and -heterotrophic features. Wassink & Manten (89) attempted in vain to isolate an obligately au-

totrophic purple sulphur bacterium from mud. Sulphur springs might furnish more interesting crude material. Manten (90) established conditions of development for large cells, answering the diagnosis of *Chromatium Okenii*. So far, for malate-thiosulphate cultures of *Chromatium*, strain D, no method of starvation could be established to prevent the uptake of considerable quantities of hydrogen upon illumination under N_2-H_2 , at pH 8 (23). Myers (91) found the oxidative assimilation of glucose and acetic acid in *Chlorella* unaffected by illumination. This proves that the immediate product of photosynthesis is not glucose, but a slowly respirable "storage" material. Earlier, Smith (92) had obtained evidence that, in photosynthesis, sucrose and starch arise from a common precursor, and that monosaccharides are formed only subsequently. Gorham (93) found the growth of a higher plant (*Spirodela polyrrhiza*) promoted by sugars; Went (94) studied sucrose-feeding in tomatoes; of special interest seems the protective effect of sucrose against solarization [(94), p. 610].

Energy relations in photosynthesis.—In recent years the possibility has been considered that part of the energy required in the over-all process is advanced by constituents of the cell, and is subsequently restored in a reaction which need not necessarily belong to the photosynthetic chain. Thus, "waste" energies in some part of the process, via a larger or smaller number of intermediate steps, may contribute energy essential to other parts [cf., e.g. (95), p. 9; (29), p. 371; (96), p. 43]. This concept has a more distinct form in the role ascribed to phosphate compounds as energy carriers, cited (1). Gaffron considered the assimilatory mechanism in green plants and in purple bacteria to be self-supporting as to phosphorylation (96). Kok formulated this requirement from the observed suppression of oxygen uptake in the light, in *Chlorella* (30). He assumed that part of the light is used in a cyclic process:



at the side of the photosynthetic chain:



and that the phosphate bond energy formed in I is essential for the completion of II. HZ stands for an ultimate or intermediate hydrogen donor; Z is much like Gaffron's "intermediate hydrogen acceptors" or "hydroxylated substances." In addition, Kok presents interesting, but hypothetical, suggestions on the relation between chemo- and photosynthesis. So far, his basic observations

are rather isolated and strongly in want of confirmation and extension over other species.

Photosynthesis of higher plants.—Many relative studies serve ecological or agricultural purposes. For the purely scientific study of photosynthesis, higher plants are somewhat neglected, which no doubt is partly due to difficulties in methods. The absence of a multiple routine instrument such as the Warburg apparatus for the study of microorganisms illustrates the difficulty.

Richter *et al.* (97 to 102) and the reviewer (103) used the Warburg technique, respectively with one large or several small discs per vessel. The latter method enables thorough averaging of material. Probably, closure of stomata caused the carbon dioxide pressure required to overcome limitation to be unexpectedly high (103). Intact, picked leaves showed much the same photosynthetic properties as leaf discs [A. Manten, C. H. M. Braat (unpublished)]. Richter *et al.* concluded that the photosynthetic apparatus is relatively independent of the state of development of the plant. In *Helianthus annuus*, decapitation influenced physiological conditions of the leaves (99). Gabrielsen (104) found quantum yields of 0.04 to 0.08 for absorbed light in sun-leaves of *Sinapis alba*. Absorption was estimated from data of other authors. Wassink (103) reported about 0.06 for various leaves. In wheat Gabrielsen (105) found the ear to contribute considerably less to dry matter production (105) than was concluded earlier [Boonstra (106)]. Williams (107), and Watson (108) made contributions to the "net assimilation rate" concept. Romose (109) and Larsen (110) compared dry weight increase and photosynthetic rate. Hagem (111), in conifer seedlings in Norway, found dry matter increase continuing during winter. Dam *et al.* (112) found no direct relation between leaf pigments and the occurrence of vitamin K. Photosynthesis in anthocyanin-containing plants was reviewed by Blank (113). Went (94) took growth in the tomato in darkness as measure for previous photosynthesis. Only sucrose was found related to external conditions [cf. also Smith (114)]. Sucrose formation in tomato stopped early in the afternoon (115). Unlike the sunflower (116), sucrose only accounted for 10 to 20 per cent of dry weight increase in the tomato (117). Hewitt (118) found losses in dry weight during night in *Phaseolus* leaves higher than could be accounted for by losses in carbohydrate. Eyster (119) studied starch formation from carbohydrates also in chlorophyll-containing plants. Relation of winter hardiness to photosynthesis is sug-

gested (120, 121). Simonis (122) studied the effect of water supply with *Trifolium spec.* Many further studies on photosynthesis of higher plants are, e.g., in *Plant Physiology* (Vols. 15 to 22, 1940-1947).

Fundamental studies should lay stress on homogeneous material, and consider the possibility of clonal variation (123, 124). Larsen (125) studied photosynthesis in relation to polyploidy in *Solanum*.

Miscellaneous.—Isotopes were used, e.g., in studies on sugar synthesis with the aid of sucrose phosphorylase [P^{32} (126)]; on photosynthetic formation of starch [C^{13} (127)]; and of sugars and other compounds [C^{14} (128)].

Myers (32, 129) and Pratt *et al.* (130) continued their studies on *Chlorella*-cultures. According to Myers (129) the number of chlorophyll-molecules per cell being practically constant, suggests a cellular or physiological type of the photosynthetic unit, which has quite different implications from those required of a physical type of unit.

Dam (131) found vitamin K production in *Chlorella* six to ten times higher than in various Athiorhodaceae. Giersch *et al.* (132, 133) report production of a growth factor in *Stichococcus* cultures upon ultraviolet radiation. Hutner (134) studied the vitamin requirements of Athiorhodaceae; Flint *et al.* (135) reported on antibiosis in blue-green algae; Rice (136) described a culture method for *Volvox aureus* Ehr. Österlind (137) studied the growth of *Scenedesmus quadricauda*. Pringsheim (138) collected his experience on algal culture in a concise monograph. Alg  us (139) studied extensively nutrient requirements in Chlorophyceae. The finding that sucrose, contrary to glucose, galactose, and maltose, could not be used at all seems important.

Tseng & Sweeney (140) studied photosynthesis of *Gelidium cartilagineum*, with special reference to the carbon dioxide factor. Free carbon dioxide, rather than bicarbonate ion, determined the rate of photosynthesis. Photosynthetic and respiratory quotients hardly differed from unity. Steemann-Nielsen (141) found bicarbonate ions to serve directly as a source of carbon dioxide for some submerged plants, whereas they had hardly any effect with some others (142). He found bicarbonate ions transported transversely through a leaf of *Potamogeton lucens* (143). B  nning & Herdtle (144) studied photosynthesis of thermophilic blue-green algae with the Warburg technique. Curiously, they used a carbonate buffer

with potassium hydroxide in the side arm. This may well confine the readings to pure oxygen production, but, no doubt, will rapidly disturb the buffer causing strong changes in the alkalinity of the medium.

Blinks & Lewis (145) studied induction effects (flashes of 0.001 sec.) with rapid carbon dioxide- and oxygen-recording methods. Wassink & Kersten (11) linked up photosynthesis and fluorescence at various light intensities during light periods of the order of seconds. Steemann-Nielsen (146) studied long-time induction phenomena with *Fucus*. The reviewer does not fully appreciate his theoretical derivations.

Brown (147) reported a starch-like product formed as principal carbohydrate by *Scenedesmus obliquus* in continuous light. Sucrose, too, was appreciable. Foster (148) failed to confirm the supposed occurrence of a formaldehyde-polymerizing enzyme in leaves. Plant carbonic anhydrase received renewed attention (149, 150). Among studies on plastids and chlorophyll formation (151 to 158), Frey-Wyssling & Wuhrmann (157) proved the earlier supposition that the "quadrant effect" of chloroplasts in polarized light is due to optical lens failures. Jungers & Doutreigne (158) found chlorophyll located exclusively in discoidal grana (0.7 to 1.4 μ diam.) in greening amyloplasts of potatoes and in some other chloroplasts. Frank (159) showed the action spectrum of chlorophyll formation to be closely similar to the absorption spectrum of chlorophyll, pointing to a pigment precursor ("protochlorophyll") closely related to chlorophyll. In *Lepidium*, Braat obtained preliminary indications for two dark processes in chlorophyll formation, one limiting greening at high light intensities, the other after long periods of illumination (unpublished). Okunzov (160) found copper sulphate sprays to increase chlorophyll content and to prevent the "physiological ageing" of chloroplasts. A few studies on carotenoids of assimilatory tissues are listed (161, 162) in addition to those referred to already. Luck & Strain (163) made suggestions on nomenclature. The colorless, fluorescent carotenoid phytofluene (164) seems not to be found in assimilatory tissue. Strain (165) discussed the sequence of pigments in adsorption columns.

Spoehr (166) reported important work on various aspects of photosynthesis. Westenbrink (167) reviewed the work of the Utrecht-Delft group during the war, including some unpublished observations.

Model studies.—The work of Baly (168) will not find ready appreciation by workers on "biological" photosynthesis (24). Nevertheless, it seems worth while to mention that, recently, van Rysselberghe *et al.* (169) report a (nonphotochemical) formation in small amounts of a C_6 -compound from carbon dioxide in the surface phase of the drops of a dropping mercury electrode, and they state:

The conditions existing at the surface of the mercury drops in the polarograph may have significant points of similarity with conditions in plant leaves.

The future will have to teach us how far indeed these relations are significant. Terenin & Kariakin (170) described a reversible proton transfer in a sublimated composite film of an organic acid and acridine in which the proton shift from the acridinic cation to the acid anion is caused by light, whereas the reversal is spontaneous. Reactions of this type well deserve our attention even though this is not presented as a model study of photosynthesis.

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MINERAL NUTRITION OF PLANTS¹

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Though a great number of papers on the mineral nutrition of plants appear every year, relatively few are of general theoretical interest. Most contributions deal with questions of practical and locally restricted importance. Some problems, viz., the mechanism of ion absorption and the demand of the plant for trace elements, have been attracting interest for several years. To some extent, agreement has been reached on the essentials of both problems. It would therefore be natural for attention to be focused upon the functions of the elements, but progress on this point is remarkably slow. Research at present is obviously not concentrated on any dominant question, which may be due to a lack of essential biophysical and enzyme-chemical methods. The above subjects were reviewed by Lundegårdh (55); recent contributions as well as some problems of mineral transformations in the soil that are of immediate physiological interest are summarized below.

AVAILABILITY OF MINERAL NUTRIENTS IN THE SUBSTRATE

As it has been shown that ions adsorbed on colloids may be as readily available to plants as ions free in solution [see Arnon (1), Richards (68)], possibly by contact exchange with the roots, the question of the actual state of the ions in soils and artificial nutrient media deserves special attention, even though, as Lundegårdh has pointed out (55), contact exchange differs quantitatively, not qualitatively, from the uptake from a solution.

Artificial colloidal media.—The method of incorporating artificial colloids carrying nutrient ions in sand cultures has been studied by Wittwer *et al.* (88); one of the advantages stressed is that nutrient ions may be added without changing the osmotic concentration of the substrate, and without the addition of soluble ions of the opposite sign. One essential condition is that the colloid must be chemically inert, which is the case with the tested "Zonolite."

In order to find an entirely synthetic colloidal medium, Arnon & Grossenbacher (2) tried Amberlite resins as nutrient substrates. Amberlites are manufactured for anion as well as for cation ex-

¹ This review covers the period from October, 1946 to September, 1947.

change. The authors prepared Amberlites containing one single nutrient ion each, and mixed them with sand into a medium in which all mineral nutrients were in adsorbed condition. Tomato plants failed to grow normally in this substrate, and leachates contained only traces of readily available calcium and magnesium. Where the potassium and nitrate Amberlites were replaced by a potassium nitrate solution, the plants made good growth, and the leachates held appreciable amounts of calcium and magnesium. The plants can apparently not utilize calcium and magnesium from the colloid, although these ions are easily replaced by potassium. The authors come to no definite conclusion for or against a root-colloid contact exchange mechanism. In a later communication Arnon & Meagher (3) emphasize that the degree of saturation of calcium is the factor determining its availability.

The absorption of potassium by corn plants growing in natural soils and in artificial colloids prepared with varying degrees of potassium saturation has been studied by Ratner *et al.* (65), who tried to determine the relative importance of contact exchange by isolating the roots from the colloid suspension by means of colloidion and parchment bags. Several objections may be raised to this procedure, but the authors come to the conclusion that contact exchange becomes relatively more important with decreasing mobility of the potassium. In soil saturated with 50 per cent potassium and 50 per cent sodium, about 45 per cent of the absorbed potassium is taken up by direct contact; if there is 50 per cent potassium and 50 per cent calcium the corresponding figure is 20 per cent, etc. In the writer's opinion these figures do not prove anything, since the amount of potassium taken up without contact must necessarily decrease with decreasing mobility, and no absolute figures for the absorption are given.

Potassium fixation.—Potassium occurs in the soil in three forms, viz., as easily exchangeable potassium (released by dilute salt solutions), as fixed potassium, which may be released by 0.5 to 1.0 *N* hydrochloric acid, and as difficultly available or nonavailable potassium forming part of the primary or secondary mineral compounds. Attoe & Truog (5) grew corn and oats in a silt loam deprived of the first fraction, which still yielded 62 per cent of the crop from the intact soil, but when the fixed fraction was extracted the yield decreased to about one-fifth of the normal. The fixed fraction is thus rather easily available to the plants. Stewart & Volk (78) depleted ten soils of exchangeable potassium by con-

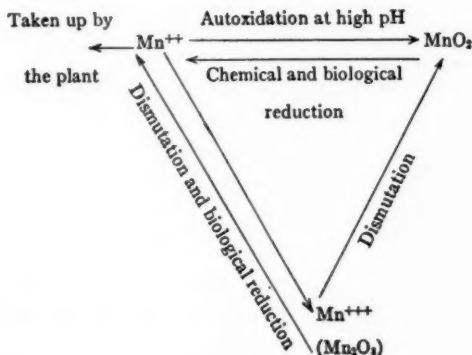
tinuous cropping, and analyses of the crops showed that, on an average, two-thirds of the utilized potassium came from non-exchangeable forms.

The nature of this fixation is of course wholly a soil chemical problem, but it is of physiological interest in connexion with the contact exchange mechanism. Several explanations have already been given; in recent papers Joffe & Levine (40, 49) claim that the fixation is reversible, and specific to the rather similar potassium and ammonium ions. Martin, Overstreet & Hoagland (57), on the other hand, state that potassium, rubidium, caesium, and possibly hydrogen ions are fixed by changing from a reversibly adsorbed condition to a nonexchangeable form, while other alkali metals, including ammonium and alkaline earth ions, are not. They showed that the organic matter in the soil is not responsible for the fixation. In general the authors seem to agree on the opinion advanced by Truog & Jones (82) that potassium enters the mineral particles in empty places in their silicate lattices. Fixed potassium can be partly released by lime. York & Rogers (90) try to explain the divergent opinions on this action of calcium, which sometimes results in an increase, sometimes in a decrease of available potassium. They studied this action in six soils of very different properties, and identified two soil-chemical actions of calcium. Lime will partly release nonexchangeable potassium, but if more potassium is added together with lime, part of the potassium will become fixed. Soil analyses show only the net result, which may be positive or negative with regard to the amount of available potassium. The investigation is not concerned with whether this is due to two different modes of potassium fixation, or to lime shifting an equilibrium between fixed and easily exchangeable potassium. Joffe & Levine (41) claim that the increased fixation is due to hydrogen ions and not to the lime itself. In addition to these actions, there is the physiological effect of calcium decreasing the utilization of potassium, which must also be taken into account when considering crop production. Another form of potassium fixation, involving biological fixation in microorganisms, has recently been studied by Hurwitz & Batchelor (38). In their experiments, a silt loam mixed with organic manure fixed up to two hundred pounds of potassium per acre in a form nonleachable by 1 *N* ammonium acetate. How this would work out quantitatively under natural conditions is not known, but one may expect to find pronounced seasonal fluctuations.

Manganese.—A series of interesting papers deal with the biological and nonbiological transformations of manganese in soils and its availability to plants. Only a minor part of the soil manganese occurs in the form of exchangeable Mn^{++} , the rest forms insoluble oxides, especially above pH 7. Only Mn^{++} is directly absorbed by the plant, but the oxides are available in so far as they are easily reduced and transformed into Mn^{++} ; this fraction is called reducible manganese, and was determined by Leeper (46) by extraction with a neutral hydroquinone solution. Our knowledge of manganese transformations has been considerably increased by the following investigations. Heintze (31), reporting on the relation of manganese in the soil to deficiency in crops, states that there is no close connexion between manganese deficiency and exchangeable or reducible manganese, the latter being extracted by 1 *N* calcium nitrate and 0.2 per cent hydroquinone. Deficiency appears at low manganese contents as well as at high nitrifiable nitrogen contents, which confirms the assumption of a connexion between manganese and nitrogen metabolism. Manganese toxicity is found in acid soils, in which according to Hale & Heintze (28) exchange of 13 to 41 per cent of the reducible plus the exchangeable manganese may be obtained, while in normal soils of pH 6.0 to 6.5 only a small part is of the latter form. In such cases the manganese content of the vegetation is ten to one hundred times above normal. Heintze & Mann *et al.* have devoted a series of papers to the availability of the different manganese fractions. They found (33) that salts of poly- and hydroxycarboxylic acids, such as citrate, tartrate, malate, and salicylate, will form complex Mn^{++} and Mn^{+++} compounds with manganic hydroxide, soluble even at an alkaline reaction and in natural soils. The same is the case with pyrophosphoric acid [Dion & Mann (17)]. Bremner and co-workers (10) extended these studies, and found in the tested extracts an obvious parallelism between manganese and iron on the one hand and organic compounds from the soil on the other. They propounded the hypothesis that the heavy metals are partly fixed in the soil in insoluble organic compounds from which they can be released by solutes forming strong complexes. Heintze & Mann (32) showed by analyses that the presumed organic complexes contain Mn^{++} , and postulated that the usual occurrence of manganese deficiency in organic soils is due to a similar fixation of divalent manganese in organic complexes. Dion, Mann & Heintze (18) have tried to identify the easily

reducible component of the soil manganese. The reducing systems applied by them were calcium nitrate or ammonium acetate together with hydroxylamine or hydroquinone over a wide range of pH. The results show, briefly, that MnO_2 belongs to the easily reducible fraction, and that in soils containing little reducible manganese, most of it will occur in oxides other than MnO_2 .

Mann & Quastel (56) have also studied the manganese cycle in the soil by means of perfusion experiments, in which the soil conditions can be carefully controlled and the biological processes proceed undisturbed [for the methods, see Lees & Quastel (48), Audus (6)]. A more complete record of the same results is given by Quastel (63). Under aerobic conditions and at pH 6.0 to 7.9 Mn^{++} is oxidized microbially to Mn^{+++} [see also Dion & Mann (17)]. This assumption is supported by the fact that the rate of oxidation follows a logarithmic curve, indicating the action of a proliferating microbe; a medium concentration of manganese produces a maximum rate of oxidation; the reaction ceases if the soil is heated to 80° to 100°C ., and is inhibited by cell poisons like sodium azide, sodium iodoacetate, and chloretone. A nonbiological oxidation takes place above pH 8. MnO_2 may, on the other hand, serve as a hydrogen acceptor in the presence of a hydrogen donor and a suitable carrier. Experiments were carried out with propionic acid bacteria and pyocyanine as a carrier. Glucose may serve as a hydrogen donor to reduce MnO_2 to Mn^{++} in the soil. The Mn^{+++} formed will then in its turn be dismutated nonbiologically to Mn^{++} and MnO_2 , completing the cycle. Of all forms of manganese, Mn^{++} alone is directly available to the plant according to the scheme:



Anaerobically, the complicated equilibria will for obvious reasons tend towards Mn^{++} . These investigations give a fairly complete picture of the known transformations of manganese. The biological processes may of course not be uniform, but proceed along different lines, and the scheme ought also to include the frequently encountered precipitation of manganese on the surface of both higher and lower plants, as well as the fixation of divalent manganese in organic complexes mentioned above.

THE MECHANISM OF ION ABSORPTION

The use of labelled radioactive elements in physiological and biochemical research work is a great advance in method, which has been used in several investigations of ion absorption. The assortment of radioactive isotopes is as yet restricted. The isotope P^{32} , with a half life time of 14.3 days, is most commonly used in recent works by Hevesy (34), Comar & Neller (15), and Overstreet & Jacobson (62). Another rather stable ion is Rb^{86} , half life 19.5 days, which is used in pure form together with P^{32} by Overstreet & Jacobson without inert carrier in a concentration of $10^{-9}M$. The alkali isotopes K^{42} and Na^{24} (36), which are more unstable, however, with half lives of 13.5 and 15.5 hours, are of greater physiological value than rubidium. Wernstedt (87), finally, has introduced a heavy isotope, thorium-B (radio lead, half life 10.6 hours), into studies of this kind.

So far, these investigations are of interest mostly from the point of view of method, and they have supported the generally accepted conceptions of the absorption processes obtained by experiments with inert electrolytes. The most apparent advantage of the use of radioactive tracers is that it permits a rapid determination of ion absorption at short intervals of time. Several of the papers quoted confirm the assumption that the first step of the absorption is an adsorption of the ions to the cytoplasm—a non-metabolic exchange of ions [cf. Lundegårdh (55)]. Wernstedt (87) claims to have shown that lead is temporarily combined with the cytoplasm granules, because both respond to centrifuging by accumulating at the bottom of the large *Chara* cells used in her experiments. This confirms a similar statement by Mullins (59) as to the distribution of K^{42} . Holm-Jensen, Krogh & Wartiovaara (36) followed the absorption of potassium and sodium in the large cells of *Tolypellopsis* and *Nitella* for 120 hours, and found an

accumulation in the cytoplasm in the first hour, followed later by a levelling of the differences in concentration of the cytoplasm and cell sap. In order to minimize respiration and other metabolic processes, Overstreet & Jacobson (62), working on isolated barley roots, studied the absorption of rubidium and phosphorus at 0°C. The resulting nonmetabolic absorption of rubidium proceeds rapidly to an equilibrium, while the rate of absorption of phosphorus is slower and constant. The absorbed rubidium is easily replaced by inert rubidium, but the absorbed phosphorus is difficultly replaceable. Very similar results were obtained by Stiles & Dent (79) in experiments on the absorption of manganese chloride, using tissue discs of carrot, mangold, swede, and beetroot. The absorption of manganese began rapidly, continuing at a slower rate after a more or less pronounced lag period, while the absorption of chloride proceeded very steadily. The lag period was reduced by washing the discs in aerated water, which in earlier experiments had proved to increase the respiration. The first phase of the cation absorption was identified by the authors as an adsorption, conditioned by and possibly developing into Donnan equilibria. This was followed by active absorption. Stiles & Dent emphasize that the first phase of the absorption can only be demonstrated if the metabolic level is so low that there is a distinct lag period between the two phases. The nonmetabolic phase nevertheless must be assumed always to occur as an initiation of the absorption of every ion.

Holm-Jensen *et al.* (36), with the aid of K^{42} and Na^{24} , verified the assumption that the active mechanism of ion absorption is localized in the outer protoplasmic boundary [cf. Lundegårdh (55)]. They also pointed out one source of error in ion absorption experiments, viz., that the alkali cations are strongly adsorbed in the cell wall, and in short-time tests, e.g., with radioactive ions, the real absorption of ions by the cell can therefore not be accurately determined in the usual manner from the decrease in the ion content of the external solution provided. The introduction of short tests, using often very dilute solutions, which is facilitated by the use of radioactive elements, has obvious advantages, but may also introduce new sources of error, which must be taken into account and eliminated.

The influence of the oxygen tension on the absorption of water, nitrogen, phosphorus, potassium, calcium, and magnesium, was

investigated by Chang & Loomis (13), who used wheat, corn, and rice in solution cultures as test plants, the last-named normally developing under conditions of oxygen deficiency in the substrate. If carbon dioxide was bubbled through the nutrient solutions, the total absorption of water and mineral nutrients decreased strongly, potassium being even exuded into the substrate. The intake was reduced in the order $K > N > P > Ca > Mg$, the absorption of calcium and magnesium paralleling that of water. Rice was no exception. That this was not due to a lack of oxygen is indicated by the rather insignificant effect of excluding oxygen by covering the surface of the solutions with paraffin or by bubbling nitrogen through them, which somewhat increased the water uptake. Only the absorption of potassium and phosphorus was significantly lowered in these cases. Nothing is said about the quality of the nitrogen used, and commercial nitrogen always contains oxygen. The authors explain the effect of carbon dioxide as a direct toxic action produced by changes in the internal pH and the subsequent formation of some carbon dioxide-protein compound. This problem is certainly of interest, but in view of the current theories regarding ion absorption, further theoretical interpretation ought to be withheld until definite information is available on the changes in pH and the electrical charge of the roots resulting from the carbon dioxide treatment. The same problem was tackled by Lawton (45) in another manner, viz. by growing corn in soils of different moisture contents and varying degrees of compactness, or with forced aeration. All treatments reducing the air supply decreased the ion absorption, and in every instance potassium was most affected. In spite of the rather different technique used, the other nutrients followed in more or less the order quoted above, which should be noted, though no interpretation has yet been offered.

Only three other papers dealing with ion absorption will be mentioned here. Hevesy (34) studied the absorption of P^{32} in wheat plants, and confirmed the general trend of the process and also that phosphorus is easily exuded by the roots; he agrees with Lundegårdh in assuming that the exudates contain nucleic acids.

Osterhout (61) has published a second part to his review of the absorption of electrolytes in large plant cells, in which he explains the phenomena of ion absorption and accumulation by the assumption of a nonwatery cytoplasmic membrane allowing only molecules, but no ions, to penetrate. As a matter of fact, this view is

incompatible with the results obtained from other experimental materials [Chapman (14), Lundegårdh (55)].

A rather speculative interpretation of the contents of mineral elements in plants was presented by Cooper, Paden & Garman (16). On the basis of the average potassium, calcium, magnesium, manganese, iron, phosphorus, and nitrogen contents of some twenty different plants, they emphasize that the strongest ions occur in the largest amounts, and conclude that

these data may be considered as illustrating the general tendency for plants to absorb ions selectively according to relative strength rather than the relative concentrations of ions in the nutrient medium.

The correlation is indubitably true, but their conclusion implies a half-truth. In fact, both the state and frequency of the element in the soil and the mechanism of ion absorption can be traced back to the physical properties of the elements, but a simplified formula that does not consider the details of the processes is of little significance.

ESSENTIAL NUTRIENT ELEMENTS

The generally accepted view is that the trace elements, iron, manganese, copper, zinc, boron and molybdenum, are indispensable to most higher and lower plants, and new records proving their essentiality are of more interest as regards the demand of the species in question than as to the principle. Only with respect to molybdenum does there seem to be some doubt. Warington (85) has investigated this question with lettuce, comparing the actions of molybdenum, chromium, titanium, zinc, and vanadium at 0.1 p.p.m. The absence of molybdenum decreased the yield to 60 per cent, while the other elements had very little or no effect. The results vary rather much; however, sometimes no effect was produced even by molybdenum, and seasonal though irregular variations of the sensibility of the plants were observed. Steinberg (76) has continued his investigation of the nutrient demands of *Lemna* on the same extensive experimental plan as with *Aspergillus*. *Lemna* undoubtedly demands iron, manganese, molybdenum, and boron, but the effects of other microelements are uncertain and irregular. Since growth in autotrophic cultures is limited by photosynthesis, the greatest responses were obtained in mixotrophic cultures to which sucrose was added. In this case the following growths in per cent of the maximum yield were obtained when one

of the elements was lacking: zinc, 47 to 74 per cent, copper, 35 to 81 per cent, molybdenum, 35 to 60 per cent, gallium, 87 to 94 per cent, and boron, 26 to 91 per cent. The decrease in yield is irregular even without boron, and the effect of gallium is scarcely convincing, though Steinberg considers it indubitable. It may be mentioned that *Citrus* did not respond positively to gallium or indium in experiments of Liebig, Vanselow & Chapman (50).

Steinberg (77) has studied a partial substitution of potassium and magnesium by homologous alkali and alkaline earth cations respectively in *Aspergillus*. If the supply of these ions is below 50 per cent of the optimum, the deficiency may be partly or wholly met by the next lighter homologues, sodium or beryllium. These ions can increase the yield by up to 22 per cent. By doubling the concentration of the macroelements, the maximal effects of sodium and beryllium were increased to about 70 and 50 per cent respectively, and the heavier homologues also favored growth. The same applies if the concentration of microelements or the acidity are increased. Steinberg claims that this unspecific action is due to an antagonizing effect of the excess of the elements; that there actually is such an action is obvious. The balancing of the ions seems to be of primary importance in these experiments, and the distinction between specific and unspecific actions somewhat arbitrary. The effects of sodium and beryllium are said to be specific, however, and possibly signify a real partial substitution. In view of the known function of magnesium as an enzyme activator, its replacement by beryllium is worth further attention. That potassium can be replaced by sodium and even by rubidium was already known [cf. recent records by Harmer & Benne (29), Holt & Volk (37)]. Dyer (20), studying the development of peanut seedlings, gives new examples of partial substitution of sodium for potassium; the latter retards the respiration and utilization of proteins due to an unspecific stabilizing action on the cytoplasm. Sayre & Vittum (71), comparing the action of sodium on soybeans and beets, emphasize that with the latter crop sodium not only replaces potassium but is favourable in itself. This leads to the still unsettled halophyte problem. Another problem of substitution is that of strontium for calcium. Lundegårdh (54) studied the growth of root hairs with the aid of microfilm; below pH 4.3 this was nil, except in the presence of calcium, which illustrates the known power of Ca^{++} to antagonize hydrions. This function may be

taken over by strontium, which is reasonable, considering the similarity of the ions, but prolonged action produced strontium poisoning.

Isolated root tips growing in synthetic media are generally assumed to need the same mineral elements as intact plants. This problem has not been thoroughly investigated with regard to the trace elements, and conflicting statements are found in the literature. White early emphasized the demand for iodine of tomato roots, and Hildebrandt *et al.* (35) obtained positive effects of iodine also on tissue cultures. Glasstone (27), investigating in particular the demand of tomato roots for trace elements, grew them successfully on macronutrients, vitamins, sucrose, iron, and copper, but without manganese, molybdenum, zinc, and boron. The amounts of the latter four elements did not exceed 0.0001 p.p.m. Much more work is necessary for a definite settlement of this important but technically difficult problem.

Drobkov (19) makes the surprising statement that peas, even if inoculated, do not form nodules in the absence of radium. Radium was supplied in concentrations from 10^{-10} to 10^{-8} gm. per liter. The nonformation of nodules corresponds to an inability to fix nitrogen. Each culture was given 0.36 gm. nitrogen in bound form, no increase was observed without radium, but with radium 0.33 to 0.35 gm. nitrogen was fixed. These interesting results ought to be verified on other legumes.

In this connexion it is pertinent to mention two records of the ecologically rare occurrence of actual deficiency of calcium [Thorne (80), Bower & Turk (9)], which is met with on alkaline soils with a pH of 9.6 and a 40 per cent saturation of the soil with sodium. Calcium deficiency, obviously due to insufficient absorption in the presence of an excess of alkali cations, may occur even when the soil contains lime [cf. (3)].

EFFECTS AND FUNCTIONS OF THE ELEMENTS

As already noted, progress is very slow in this field. The actions of the elements are seldom studied biochemically, and usually only a superficial correlation of the content of a certain element to some plant function is shown. It is often impossible therefore to decide whether a given correlation is causal or not.

Some summaries have recently appeared. Brenchley (11) has published a supplement to an earlier review of the minor elements,

with bibliography up to 1945. Interrelations of macronutrients have been summarized by Beeson (7) with special regard to the influence of fertilization on the contents of different mineral elements in the plants as well as on the quality of the crops. It is inherent in the subject that this paper is a compilation of a vast number of data of very varying nature.

Macroelements in general.—The influence of macronutrients on the vitamin contents of plants has been treated mainly from a more or less practical point of view. Various records have been given of the influence of mineral elements on the concentrations of carotene and ascorbic acid. Bernstein, Hamner & Parks (8) found no effect from supplying potassium, calcium, phosphorus, nitrogen, magnesium, or sulphur to turnips, and, according to Sideris & Young (73), potassium does not affect the ascorbic acid contents of pineapple; this applies also to different legumes [Ferres & Brown (23)]. On the other hand, Watson & Noggle (86) report a significant increase in ascorbic acid in oat leaves deficient in potassium or magnesium, but no effect of calcium, sulphur, nitrogen, or phosphorus. All nutrient deficiencies were associated with low riboflavin content. The conflicting statements, especially with regard to ascorbic acid, may be interpreted as indicating that no direct connexion exists, nor have any reasons been advanced in favor of a causal relation. This does not detract from the importance of the practical aspects of this nutritional problem. [As to vitamins and trace elements see Ferres & Brown (23), Lo & Chen (52).]

Sideris & Young (73) have further studied the influence of potassium on the nitrogen metabolism in pineapple and found high protein and little soluble nitrogen when much potassium was supplied. They conclude that potassium regulates the formation and decomposition of high polymers in the cells by means of its general effect on cytoplasm activity, which may imply a colloid chemical action of potassium, supposed to be the main function of this element in the cell [see also Dyer (20)].

Phosphorus.—With respect to phosphorus, some papers record determinations of various phosphate fractions which are of interest in judging the functions of the element in plants. Heard (30) has divided the phosphorus in barley plants into four fractions; (a) inorganic phosphorus, of varying amount, but usually averaging

70 per cent of the total; (b) labile esters, hydrolyzed by 2 *N* hydrochloric or sulphuric acids in seven minutes, constituting 0 to 10 per cent: this fraction is supposed to comprise mainly adenyolphosphoric acid; (c) resistant esters, constituting about 20 per cent. They contain the sugar esters functioning in respiration, but no triosephosphate could be detected. This fraction was divided into two: (i) one hydrolyzed in 180 min., containing phosphopyruvate and hexosediphosphate in hardly significant quantities, and the other (ii) the finally remaining unhydrolyzable fraction, constituting the main part of the resistant esters and involving hexose-6-phosphate. The active respiratory phosphates hardly occur at all, but the authors emphasize that the analyses give only a picture of the momentarily existing phosphate reserves and naturally do not show the rates of transformation of the different fractions. Sokolov (75) studied the relations between inorganic and organic phosphorus in corn at varying levels. When these were low, an increase merely promoted growth, but did not change the concentration of any of the fractions; when they were supraoptimal, only the inorganic but not the organic phosphorus content increased. The picture is what might be expected from an element which at minimum levels serves as a first-rate growth factor, and in the event of luxury consumption is stored in a physiologically inactive form.

The importance of mycorrhiza to the phosphorus nutrition of conifers has been investigated by McComb & Griffith (58). Previous workers on the subject have denied any such importance [see Richards (68)], but McComb & Griffith claim that the stimulation of root development is connected with increased absorption of phosphorus, though this is not the sole role of the mycorrhiza. Under certain conditions uninoculated plants of Douglas fir suffered from phosphorus deficiency, whereas inoculated plants increased their phosphorus content even without any additional external supply. The influence of phosphorus on the root growth of wheat plants has been studied by the writer (12). In concentrations exceeding starvation, phosphorus only increased the rate of cell divisions (probably by accelerating the cytoplasmic growth) but not the actual rate of cell elongation. In these respects the action of phosphorus is quite the reverse of that of nitrogen, supplied as nitrate, which when given in excess pronouncedly increases

the rate of cell elongation. This illustrates the importance of a proper balance of the nutrients for an optimal and infinite development of the roots.

Sulphur.—The utilization and transformations of inorganic sulphur compounds may be included in the subject of mineral nutrition, though its further metabolism falls beyond that subject, alongside the nitrogen metabolism. As to sulphur auto- and heterotrophy, a series of interesting papers have appeared dealing with the properties of x-ray mutants of lower plants. Working on *Ophiostoma multiannulatum* among ninety-four mutants obtained by x-irradiation, Fries (24, 25) found thirteen unable to utilize sulphate. Such forms have been designated parathiotrophic to distinguish them from the normal euthiotrophic (sulphur autotrophic) forms. The parathiotrophic forms will assimilate cysteine and cystine, but have lost the property of reducing SO_4^{2-} . Five of the mutants could adapt themselves to sulphate nutrition and regain their lost property. This is a parallel to the deficiency mutants induced by x-rays in *Neurospora*. The sulphur mutants offer valuable material for studying sulphur assimilation, which is very incompletely known. Lampen *et al.* (42, 43, 44) subjected *Escherichia coli* to such an investigation. *Escherichia* behaves like *Ophiostoma* in that x-irradiated mutants may become parathiotrophic, and that the mutations obviously involve the loss of certain enzymes. Two *Escherichia* mutants were unable to reduce SO_4^{2-} to SO_3^{2-} , one to reduce SO_3^{2-} further to S^{2-} , and four could not convert homocysteine into methionine. Other mutants with less distinctive properties were also obtained. Further investigations in this field are awaited with interest.

Magnesium.—A whole number of *Soil Science* (Vol. 63, No. 1) has been devoted to magnesium in soils and plants. It opened with a summary by Zimmerman (91) of the known facts of the functions of magnesium in plants. An original aspect of the magnesium requirements of plants was presented by Eisenmenger & Kucinski (21). They grew a number of plants of different families with and without magnesium fertilizers on natural soils, and claim to have found that more high developed plants are more resistant to magnesium deficiency than the more primitive plants, such as members of the Ranales, Magnoliaceae, Anonaceae, and others. Highly developed plants are characterized by "greater sturdiness" and are "far more resistant to abnormal agencies," also to mineral

deficiency in general, not especially to lack of magnesium. This aspect is doubtlessly interesting both phylogenetically and ecologically, but the results are very incompletely reported, and the authors do not even make it clear on how large a material their conclusions are based. They do point out, however, that exceptions occur.

An increased utilization of phosphorus is often obtained by supplying dolomite or magnesium silicates. Truog and co-workers (81) have confirmed this by field and water cultures of peas. The addition of dolomite or magnesium sulphate will increase the phosphorus contents of the crops more reliably than an increased phosphorus supply (cf., the well-known similar action of lime). The explanation given is that magnesium acts as a "carrier" of phosphoric acid. No comment on this term or of the mechanism involved is provided, but the authors discuss the practical importance of the phenomenon. The conditions are no doubt related to the fixation of phosphate in soils, a problem of which too little seems to be definitely known to warrant presentation here. Other papers in this magnesium series deal mainly with applied problems relating to the magnesium requirement of *Citrus*, apple, and tobacco.

Iron.—The relation of iron to chlorophyll formation was studied by Jacobson (39) on peach leaves suffering from lime-induced chlorosis. Contrary to earlier statements [see Chapman (14)], he records an obvious connexion between chlorosis and the iron content of the leaves. Asana (4) on the other hand showed that rice becomes chlorotic with nitrate as the source of nitrogen at pH 6.0 but not with ammonia, either at pH 6.0 or 4.7. In this case iron content was as high or higher in chlorotic leaves than in normal ones [cf. (74)]. This emphasizes the complex nature of the chlorosis phenomena. Jacobson confirms the expediency of dividing the total iron into two fractions [Oserkowsky (60)], one inactive, which is the minor portion and is not involved in the chlorophyll formation, and the other an active fraction. The latter increases with increasing chlorophyll contents. There is a parallelism between total iron and iron soluble in 1 *N* hydrochloric acid, and the part insoluble in acid is fairly constant irrespective of the chlorophyll contents. Thus the active fraction is included in the acid-soluble part. In tobacco leaves the active iron is localized in the chloroplasts, and any excess of iron absorbed accumulates outside them.

In this connexion it may be mentioned that Sideris & Young (72) studied the influence of iron on various nitrogen fractions of pineapple at ammonium and nitrate nutrition. They found that the addition of iron increased the amide and amino nitrogen contents with ammonium nutrition, and the peptide and protein nitrogen contents with nitrate nutrition. These results are of interest primarily with respect to the nitrogen metabolism, but they are also interpreted as indicating that iron takes no direct part in the nitrogen metabolism, its action being probably restricted to regulating chlorophyll formation, and hence to the supply of carbohydrates. Lin (51) grew rice in aerated and nonaerated solutions containing different sources of iron, supplying nitrogen in the form of nitrate or ammonium, and studied the appearance of chlorosis. His experiments show without exception that chlorosis never appears in ammonium cultures [see also Asana (4)], and in nitrate cultures only when the solution is stagnant, but not when it is flowing. This agrees with the practical experience that chlorosis will appear in naturally grown rice fed with nitrate. Air or nitrogen bubbling through the cultures did not prevent chlorosis, nor did the addition of iron as tartrate [Lin (51)]. Chlorosis was effectively prevented, however, by the addition of 50 mg./l. sodium thioglycollate (sodium mercaptoacetate); this substance is a strong reducing agent, but whether this or some other property is involved, remains an open question. It is even uncertain whether the chlorosis is in this case connected with iron at all, though this seems probable.

The red pigment of *Torulopsis pulcherrima* contains iron, and the color of the cells depends upon the increasing supply of iron. Roberts (69) shows, however, that in this case iron only prevents the diffusion of the pigment or its precursor from the cells, but that it does not influence the real production of the pigment.

The reduction of Fe^{+++} to Fe^{++} in the soil is a bacterial process, very imperfectly known in spite of its common occurrence and general importance. The metabolism of a common, strongly iron-reducing form, *Bacillus polymyxa*, was investigated by Roberts (70) and proved to equal that of *Aerobacter indologenes* [Reynolds & Werkman (67)]. Glucose is fermented to carbon dioxide, 2,3-butylene glycol, ethyl alcohol, lactic acid, hydrogen, etc. The addition of ferric hydroxide decreases the amount of hydrogen from 51

to 31 moles per 100 moles of fermented glucose; when iron is reduced the ethyl alcohol increases and the 2,3-butylene glycol decreases. The bacterium will also reduce acetate almost quantitatively to 2,3-butylene glycol. Roberts cautiously refrains from any theoretical consideration of the iron-reducing mechanism.

Heavy metal interactions.—According to the hypothesis outlined in detail by Shive *et al.* [see Chapman (14)], the oxidation level and activity of iron in a plant is regulated by the iron: manganese balance, and this opinion has apparently been rapidly accepted by others, though very little direct evidence in its favor has been produced except for the original statement by Shive, and for observations of external symptoms of deficiency and toxicity of the two elements. This is particularly apparent in a summary by Twyman (83) covering both old and modern literature on the iron: manganese balance. That these ions are antagonistic and compete in various ways in the plant is highly probable, but caution seems necessary in drawing any conclusions from the concentrations of the elements regarding their activity in redox systems. The importance of the balance of the heavy metals has been emphasized by Erkama (22), who has made an inventory of the iron, manganese, and copper contents of thirty-four wild species—fourteen from one forest area, eleven from one garden, and nine from a variety of places. The following general conclusions were drawn regarding the metal contents. Leaves rich in water have high copper and nitrogen contents, but little manganese. The manganese and copper contents generally vary inversely, though the manganese fluctuates much more than the copper, which is remarkably uniform. The manganese variations are due partly to differences in the organs of the same plant, the leaves being rich in manganese, fruits poor, and partly to the known relation of manganese to soil pH, manganese being higher in plants from acid localities. Even if these disturbances are eliminated, the manganese content varies considerably, e.g., leaves of plants from forest soil of pH 4.5 to 5.4 contained 1760 to 79 p.p.m. manganese against 31.7 to 5.5 p.p.m. copper. In perennial leaves the content of manganese increases, but that of copper decreases with age. Erkama concludes that copper is probably bound to proteins, while manganese occurs in inorganic form. Even if true, this conclusion is not based on direct observations, as the uniformity of content rather points to

copper migrating easily in the plant. Of other results, there may be mentioned the confirmation of the parallelism between ascorbic acid and manganese contents [see also Rangnekar (64)] and also of ascorbic acid and copper contents of peas in water cultures. The latter is explained by the decrease in peroxidase with increasing supplies of copper, and this enzyme is said to act as ascorbic acid oxidase. According to earlier investigations [see (55)], however, copper should increase the oxidation of ascorbic acid. Erkama (22) also concludes that copper regulates the oxidation level of iron in the same way as manganese, but direct proofs of this assumption are wanting.

Copper.—Copper is also assumed to act as a redox catalyst in the plant, and a deficiency will affect the sexual reproduction in particular. The need of copper is in some way connected with the nitrogen metabolism, but opinions diverge as to the nature of this relationship. Wood & Womersley (89) have shown that copper deficiency in oats is promoted by lack of nitrogen; copper is supposed to be bound in an immobile form in the plant [cf. Erkama (22) quoted above]. In tung trees copper deficiency causes a decrease in the apparent photosynthesis [Loustalot *et al.* (53)] leading to lower carbohydrate, especially starch and oil contents [Gilbert, Sell & Drosdoff (26)]. Copper deficiency is here associated with abnormally high protein nitrogen, which may also contribute to a lowering in the amount of nitrogen-free reserve compounds. Nothing is known about any direct participation of copper in photosynthesis, nor in the protein synthesis, but it is obvious that it is in some way involved in the complicated and labile balancing of nitrogen and carbohydrate transformations. The relation of copper to water content [Erkama (22)], which is also known to be connected with the nitrogen balance, deserves attention here. The causal connexions are still obscure, however. Quite another effect of copper was demonstrated by Lees (47) in nitrification. This is inhibited by copper poisons, e.g., potassium ethylxanthate, sodium diethyl dithiocarbamate, and salicylaldehyde. This poisonous action is neutralized by addition of copper, and to a lesser degree by iron. The result entitles Lees to conclude that copper is taking part in the bacterial oxidation of ammonia.

Zinc.—The function of the fourth essential heavy metal, zinc, is no better known than the others, apart from its occurrence in

carbonic anhydrase, but this has not yet been found in plants. Reed (66) has shown that in tomato, zinc deficiency will increase the content of reducing sugars and decrease that of inorganic phosphorus, phenolase, and phosphatase. With respect to Warburg & Christian's (84) statement that zymohexase contains copper and zinc, and that this enzyme (inactivated by cysteine) is reactivated by zinc, Reed assumes that phosphorylation is inhibited in this material. The evidence in favor of the opinion that zinc is involved in this part of the carbohydrate transformations seems to be rather strong, but it is still uncertain whether this is its only action. Several authors ascribe to zinc a similar role as a redox catalyst as iron, manganese, and copper, or as a regulator of the oxidation levels of iron or copper [for references see (55)]. This view is based on the absolutely erroneous assumption explicitly put forth in some papers that zinc, too, is a valency-changing metal, whereas it is exclusively divalent.

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PLANT HORMONES

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The subject of this review involves a large number of physiologically active compounds known as plant hormones, growth regulators, growth substances, or auxins, popular usage being in the order listed. The term "auxin" originally was applied to natural substances but has been extended to include synthetic substances which induce hormone-like responses in plants. The early use of "growth substance" has given way to "growth-regulating substances" since the former is extensively used to designate vitamin-like substances for microorganisms. There is a natural tendency for laymen and scientists alike to call physiologically active substances "plant hormones." This practice eventually may determine the terminology to be used.

Though van Overbeek (1) stated in his 1944 review that naphthoxyacetic acid is not an auxin because it failed to react in the *Avena* test, he admits that

Some terms such as "plant hormone" already have acquired through usage a meaning different from that which was originally intended. Such changes, which naturally occur, have to be sanctioned by general agreement in order to avoid confusion.

Skoog (2) in his 1947 report stated "the term growth hormone will be limited to such substances which are also known to occur in plants." Thimann & Behnke (3) made an extensive compilation of published work on propagation of plants by the aid of root-inducing substances with the title "The Use of Auxins in the Rooting of Woody Cuttings." They included under "Auxin" substances which are not active on the *Avena* coleoptile. A recent book by Avery *et al.* (4) is called *Hormones and Horticulture*. In the text of the book the authors refer to "synthetic hormones." Another book by Mitchell & Marth (5) is called *Growth Regulators for Garden, Field, and Orchard*. It appears that the trend is to use the word "hormone" in a broad sense to cover both natural and synthetic compounds which induce the well-known hormone-like responses when applied to plants.

The papers selected for the review appeared principally during

1947. They have been grouped under the following headings: Precursors and enzymatic activity; Flower-inducing substances; Comparative effectiveness; Formative influences; Translocation of radioactive substances; Inhibition of growth; Fruit drop; Parthenocarpy; Effect of 2,4-D on progeny seedlings; and Hormone weed control. Subjects like root-inducing hormones, effects on respiration, and many others which were well handled in previous reviews have been omitted. The use of hormone-like compounds for selective weed killing is well covered because this subject was given little attention in the previous review (2).

Precursors and enzymatic activity.—The work of Wildman *et al.* (6, 7) and Gordon (8) showing the presence of auxin protein complexes which yield auxin with hot alkali or enzyme treatments has been extended and results substantiated.

The cytoplasmic protein of spinach leaves was separated into two distinct fractions by precipitation with ammonium sulfate (9). Approximately 75 per cent of one fraction appeared to consist of a single electrophoretically homogeneous protein. This fraction contained approximately all of the bound auxin and also exhibited the enzymatic properties of a phosphatase. The bound auxin showed properties similar to indoleacetic acid.

Wildman & Muir (10) found the oat coleoptile produced an enzyme that converted tryptophane to an active substance and concluded that tryptophane plays a major role in the total production of auxin in the oat coleoptile. Wildman & Muir (10) found that no auxin could be extracted with ether at 0° C. but the amount extracted doubled with a 10° rise in temperature as in an enzymatic reaction. Tobacco ovaries contained a very active tryptophane-auxin converting enzyme, and it was concluded that the formation of active substance during ether extraction at 25°C. resulted from the enzymatic conversion of tryptophane. Similar results were obtained when cabbage, corn coleoptiles, green tomato tips, spinach leaves, and corn grains were used. These results were thought to give support to the contention that tryptophane is the principal precursor of auxin in plant tissue. In an earlier paper (7) it was suggested that trypsin is the principal auxin precursor in spinach leaves and that indoleacetic acid is a principal auxin. This statement was made without disposing of the early claims of Kögl *et al.* that "auxin a" is the principal natural auxin in plants. Be that as it may, many active extracts (unpublished data) studied by the

reviewers showed negative results when tested for indoles by the Winkler method. The active extract of corn pollen is a good example.

Gordon & Sánchez-Nieva (11) support the contention of Larsen (12) that indole acetaldehyde may be a neutral precursor of indoleacetic acid. The addition of the neutral auxin to pineapple leaf tissue resulted in increased amounts of active acid auxin, through enzymatic action. There was a parallelism between the levels of neutral and acid auxins in various tissues of pineapple. It was therefore suggested that the aldehyde can function as an immediate precursor of indoleacetic acid in pineapple.

Schocken (13) would simplify the whole problem of active auxin formation by assuming that all tryptophane-containing proteins yield indoleacetic acid upon hydrolysis. Purified proteins of spinach, chymotrypsin, fibrin, β -lactoglobulin, ovalbumin, and serum albumin all containing tryptophane yielded indoleacetic acid in their alkaline hydrolysates. Gelatin which is free of tryptophane yielded no indoleacetic acid. Schocken suggested that the indoleacetic acid alleged to be bound to proteins may be only a transformation product of tryptophane which appears during hydrolysis of the protein.

Flower-inducing substances.—Allard (14) has recently made a good review of flower-inducing substances reported by various authors, in connection with a report on his attempt to change the flowering habit of a short photoperiod variety, Maryland Mammoth tobacco, by grafting with a neutral type called Connecticut Broadleaf. His attempts to obtain translocation of flowering substances from the donor (Broadleaf) scions to the receptor (Maryland Mammoth variety) resulted in failure. He used various methods including complete defoliation of Maryland Mammoth scions, but in no case was there any indication of translocation of the factor which affects flowering. There are, however, good reasons for assuming that flower-inducing substances exist in plant tissues and efforts to isolate them should continue.

Galston (15), Tumanov & Lizandr (16), and Owen (17) have used 2,3,5-triiodobenzoic acid in an effort to change the flowering habit of plants. Galston (15) states that triiodobenzoic acid does not possess florigenic properties since it did not induce vegetative soybean plants to flower. However, it greatly augmented the flowering response due to photoperiodic induction. Morphological

responses of vegetative soybean plants to the chemical (shortening of the internodes, loss of apical dominance, epinasty of leaves, and premature abscission of apical leaves and buds) suggested that it causes auxin aberrations within the plant. That is, it in some way affects natural growth regulators which force the normal regulation of growth.

Tumanov & Lizandr (16) found that triiodobenzoic acid retarded growth of *Perilla* and caused formative effects. Alfalfa was more sensitive than *Perilla*. The authors found a variation in sensitivity during short and long days. The treatment caused a variation in the number and size of leaflets in alfalfa. Spraying with weaker solutions, 0.005 per cent, in long days brought about increased yield of alfalfa seed. Flax and sunflower species showed very notable changes in growth when treated with 0.01 per cent solution.

This concentration also caused peas to branch through stimulation of axial buds and fusing of leaflets. There was, however, no definite sign that triiodobenzoic acid could be considered as having florigenic properties.

Owen (17) treated a number of species with triiodobenzoic acid and brought about unusual distortions of leaves, stems, etc., but failed to change any organ from the vegetative to the flowering stage.

Comparative effectiveness.—Frequent mention is made of comparative activity of a given substance and its various derivatives. The variation in results of different workers may be due to the use of different test objects and methods of applying the chemicals. There is need for more standard test objects and methods. The *Avena* coleoptile is unsatisfactory because it is not sensitive or only slightly so to many important substances. The tomato plant is useful because it responds to all the known physiologically active substances and detects different types of activity. However, a standard test object may lead to different conclusions if methods are not standardized. To compare properly the effectiveness of one substance with another, it is essential to use a carrier that completely dissolves the compounds involved. For example, lanolin is not entirely satisfactory where acids and salts are compared because salts and acids are not equally soluble and neither do they move through lanolin with equal ease. The rate at which a substance leaves the carrier and moves into the treated tissue is sure to affect the end results.

Hamner *et al.* (18) reported that the esters of 2,4-dichlorophenoxyacetic acid (2,4-D) were more effective than the acid and the acid more effective than the salt. These three, of course, have different solubilities and need different carriers. The question is raised whether this fact may account for the different activity found since after entering the plant tissue, which is acidic, they all must become acids. Hamner *et al.* (18) also found that as herbicides the effectiveness of salts of 2,4-D was increased when applied in an acid solution. The results should be the same for both if considered on the basis of acid equivalents.

Murray & Whiting (19) applied 2,4-D and four of its salts to the cut surface of decapitated bean plants and made histological examinations of tumor growths at intervals up to thirty days. For all five substances there was fundamental similarity in the tissues which responded and in the type of response induced; that is, the response resembled that induced with 2,4-D. There were characteristic differences in the location of the tumors, the speed of the response, and time of maturation. The magnesium salts caused the tumors to grow near the cut surface while the acid caused tumors considerably below the surface. Other salts caused tumors between these two extremes. The authors interpreted this zonation as an expression of concentration gradient. The reviewers are inclined to agree with this interpretation, assuming that the salts diffused at different rates from the lanolin into the plant tissue. Since the bean tissue has a pH value of approximately five, the salts were converted to the acid form after entering the stem but different amounts entered in each case. The 2,4-D appeared to be too strong at the surface and was most stimulating some distance from the cut surface as it became weaker.

Again referring to the importance of carriers, Beal (20) found that histological responses of bean differed when substituted phenoxy acids were applied in lanolin as compared with Carbowax.

Lucas & Hamner (21) reported that onion juice increased the activity of 2,4-D when applied to bean plants for inhibition of growth. These authors tested garlic and other plant extracts but found none as effective as Spanish onion juice. It is not known how onion juice increases the effectiveness of 2,4-D. It may facilitate penetration of the hormone as suggested by unpublished work. This assumption could be tested by injecting substances alone and mixed with onion juice directly into the tissue. It is known, how-

ever, that many compounds and extracts when added to hormone-like compounds affect their activity.

Formative influences.—Modification of the pattern of leaves, flowers, and stems and the habit of growth is associated especially with the influence of three groups of hormone-like compounds, naphthoxy, phenoxy, and benzoic acids (22, 23, 24). Many references have been made to the resemblance of chemically modified leaves with those modified by virus diseases. Since the advent of 2,4-D as a herbicide many accidents have occurred in yards and gardens where growth was modified though only neighboring lots have been intentionally treated with 2,4-D. However, very little attention has been given as to how chemicals modify organs of plants. The recent work of Burton (25) is welcome as one of the first attempts to determine what happens to the structure of tissue to bring about these odd forms. Using the bean leaflet as a test object Burton worked out the normal structural developments and compared these with chemically induced modifications. It appears that the normal bean leaflet develops a lamina by the activity of a subepidermal marginal meristem, which produced four internal layers of plate meristem. The adaxial (upper) of these layers develops into the palisade layer and the other three produce the spongy mesophyll. The veins are initiated by divisions of rows of cells in the layer beneath the embryonic palisade. Many intercellular spaces (air) normally appear in the spongy tissue.

Using three substituted phenoxy acids Burton (25) found that these were more or less specific for given structural variations from normal. For example, 2-chlorophenoxyacetic acid inhibited the formation of intercellular spaces in the spongy tissue; 4-chlorophenoxyacetic acid inhibited the activity of the plate meristem (between the veins) and the veins became approximate with continuous parenchyma. 2,4-D brought about a progressive modification of all leaves developed after the chemical was applied. The latter compound also caused various structural modifications similar to those of both the other acids. Chlorenchymous tissue was usually confined to the margin, and cells without chlorophyll over vascular tissue gave the veins a transparent effect.

Burton (25) worked on only one species, the bean. It will be interesting to see how structures of other species respond to the same chemicals. Also, since some species (tobacco for example) show little or no modification in pattern when treated with 2,4-D

but become greatly modified under the influence of 4-chlorophenoxyacetic acid, they should make good research subjects.

In an effort to kill water hyacinth with commercial applications of 2,4-D, Brown (26) reported that a large acreage of cotton in the neighborhood was severely injured. Where the dosage of the accidental treatment was not great enough to kill the plants, abnormal leaves, flowers, and bolls developed. The leaf symptoms varied from slight modifications to severely malformed organs with parallel veins, extra long apices, and ruffled margins. The formative effects appeared on the new growth ten to fifteen days after the cotton plants were exposed.

King (27) used substituted phenoxy acids in her experiments with water hyacinth and refers to the hormone-like chemicals as "formagens." There is need for a term to cover all substances which have formative effects on plants.

Translocation of radioactive substances.—In an effort to determine movement and translocation of growth substances in plants, Mitchell *et al.* (28) used a growth-regulating substance, 2-iodo¹³¹-3-nitrobenzoic acid (INBA) labeled with radioiodine. Bean plants were found to absorb and translocate this compound more readily than barley. They considered this difference suggestive as to the relative susceptibility or resistance of species. An accumulation of INBA in the terminal bud of the bean plant caused a marked inhibition of growth. No such inhibition occurred in corn and oat plants perhaps because the monocotyledonous plants absorb less of the compound. The authors concluded, however, that the difference in susceptibility of bean, oat, and corn plants was probably due to the differences in the manner in which INBA reacts with the plant constituents in each case, rather than the amount of the chemical absorbed. The method used appears to have good possibilities for future studies involving various growth regulators. Wood *et al.* (29) using bean and berry seedlings treated with radioactive iodine arrived at approximately the same conclusion as Mitchell.

Inhibition of growth.—In semipopular articles, Plummer (30) and Smith (31) showed that with proper chemical treatment beets, carrots, turnips, rutabagas, and potatoes could be kept dormant through winter storage. Controls showed large sprouts of potatoes, and leaves of carrots, turnips, beets, and rutabagas. Using methyl- α -naphthaleneacetate mixed with talcum powder, it was found

that this preparation dusted on six varieties of potatoes prevented sprouts for four or five months when in storage at a temperature of 50° to 55°F. While there was some variation with different varieties, generally the treatment was so effective that potatoes stored over winter maintained the quality, weight, and appearance of new potatoes. This report verifies the early work of Guthrie *et al.* which need not be repeated here. Other methods of application involved atomizing with a sprayer, aerosol application, and dipping in the solution before storage. The latter methods are particularly effective on beets, carrots, turnips, and rutabagas before being stored.

A report of work in Great Britain by Luckwill (32) showed that sprouting of potatoes stored in boxes, sacks, barns, and fields can be inhibited by the use of methyl- α -naphthaleneacetic acid applied in several suitable carriers for the chemical. They stated shredded newspaper and talc were satisfactory carriers for the chemical but that dried soil was not. They recommended application of one-half ounce to three ounces of the chemical per ton of potatoes depending upon the season of treatment and the length of time they were to be preserved.

It has generally been assumed that potatoes which are to be used for seed should not be treated with esters of naphthaleneacetic acid. Pujals *et al.* (33), however, reported that potato tubers dusted with methyl- α -naphthaleneacetate preparations containing 20 mg. of the chemical per kg. of potato effectively inhibited sprouts for ninety-nine days but could be stimulated to sprout with ethylene chlorhydrin treatment. The ethylene chlorhydrin treatment resulted in more stems per hill of potatoes, but the yield in general was not as great as with controls. The authors state that the data obtained indicate that proper timing of dormancy-breaking treatment may result in yields equally as good as those obtained from normal seed tubers.

Daines & Campbell (34) reported that the use of two-thirds to one and one-third grams of methyl- α -naphthaleneacetate in dust per bushel of potatoes effectively inhibited sprouting though there was some variation with the storage temperatures. In some cases the methyl ester injured the tubers. The authors state that potato tubers treated with methyl- α -naphthaleneacetate should not be used for seed.

Hemberg (35) studied the effect of various plant growth regu-

lators and growth-inhibiting substances in relation to potato dormancy. He concluded that auxins are of no significance with regard to dormancy of potato tubers. He is of the opinion that natural dormancy is caused by a large number of natural growth-inhibiting substances occurring in the periderm layer during the rest period. These inhibiting substances in the periderm layer become greatly reduced at the end of approximately six weeks, at which time the tubers are said to be past their rest period. Peeling of the potato or any treatment which destroys the natural growth-inhibiting substance in the periderm will in his opinion break dormancy of the tubers.

Inhibition of flowering buds of fruit trees has been discussed by the present reviewers (36). It has, however, been impossible to delay the opening of flower buds without at the same time retarding the growth of leafy shoots. Until new methods are perfected or more selective chemicals discovered, treatment of fruit trees to delay flowering until danger of frost is past is not recommended.

It is a regular practice in commercial tobacco growing to remove the flower shoots from the terminal growth of the plant. This practice induces a large number of axillary shoots to appear. These suckers must be manually removed from time to time in order to insure proper maturing of the leaves.

Steinberg (37) applied five different plant growth-regulating substances to the cut surface of the tobacco plant after the flower shoot was removed. He found methyl- α -naphthaleneacetate more effective than 2,4-dichlorophenoxyacetic acid methyl ester, 2-chlorophenoxypropionic acid, or 4-chlorophenoxyacetic acid for inhibiting axillary shoots. He states that his data indicate the possibility that it may be economically feasible to employ chemical suppression of "suckers" in the production of commercial tobacco.

Fruit drop.—Since the early work of Gardner & Cooper (38) demonstrating the value of naphthaleneacetic acid (NA) for preventing preharvest drop of apples, many variations in chemicals required and methods of application have come to light. Batjer & Thompson (39, 40) showed that 2,4-dichlorophenoxyacetic acid was effective on Winesap apple where NA failed. 2,4-D, however, failed to prevent drop of Oldenburg, Delicious, and McIntosh varieties. Hoffman & Edgerton (41) showed that the value of NA, which is more generally used than any other chemical for preharvest drop of apples, varied with the apple variety and the

weather conditions. They showed that an inadequate supply of moisture reduced the effects of chemical sprays.

Stewart *et al.* (42, 43) recently demonstrated that 2,4-D can be effectively used to prevent preharvest drop of Valencia and Washington navel oranges. Eight parts per million of the chemical reduced the drop 30 to 60 per cent, and 25 p.p.m. reduced the drop 91 per cent. The latter concentration caused modification of leaves and higher concentrations caused considerable damage. Another interesting variation was the tendency of seedless navel oranges to develop seeds or seed-like structures when the trees were sprayed with 25 to 225 p.p.m. of 2,4-D.

The results of work on fruit drop show that apple varieties vary in susceptibility to a given chemical, Winesap being resistant to NA but responding well to 2,4-D. On the other hand, variety Williams responds well to NA but is resistant to 2,4-D. The basis for these variations cannot be understood with our present knowledge.

Parthenocarpy.—A large number of chemical compounds applied to open flowers of tomato plants cause parthenocarpic development of fruit (44, 45). Chemicals which do not cause cell elongation and otherwise are not physiologically active may be effective when applied to buds and flowers of tomato. Among the list are many benzoic and phenoxy acids. The most effective substance from the concentration standpoint is 2,4-D, 2 to 5 mg. per l. being effective (44, 46). This concentration may be compared with 500 to 1000 mg. per l. for indolebutyric acid or 50 to 100 mg. per l. of naphthoxyacetic acid. The method of application appears to be unimportant so long as the effective chemical in some way gets to the ovary. Howlett & Marth (47) showed that water solutions of the active chemicals, emulsions, and aerosols were effective. They used indolebutyric acid, naphthoxyacetic acid, and 4-chlorophenoxyacetic acid separately or mixed together. Applied to the first three clusters of flowers the treatment causes earlier fruit production and yield increases of twelve thousand to twenty-three thousand pounds per acre. The pulp development of the fruit varied with the location in the greenhouse. In one location the pulp development was poor but the same treatment used in another greenhouse with different temperature and humidity produced excellent fruit. The most effective treatment involved all three chemicals mixed together.

Holmes *et al.* (48) treated open tomato flowers with 75 mg. per l. of naphthoxyacetic acid and analyzed the resulting parthenocarpic fruit for mineral elements and vitamins. They found very little difference in water, carotene, or magnesium content of treated or control tomatoes. The treated tomatoes averaged slightly more nitrogen, phosphorus, potassium, and calcium than controls. The controls, however, had slightly more ascorbic acid than treated fruit. The differences did not appear to be significant.

A new use has been found for hormone-like substances to induce formation of seedless Calimyrna figs without the use of male or caprifigs. The method described by Crane & Blondeau (49) consists in spraying the trees with a solution of naphthoxyacetic acid and indolebutyric acid at pollination time. A mixture of indolebutyric acid, 1500 p.p.m., and naphthoxyacetic acid, 50 p.p.m., in water solution, or indolebutyric acid, with a range of concentration from 1500 p.p.m. to 2670 p.p.m. gave satisfactory results. Naphthoxyacetic acid was not tested alone. Compared with controls the treated trees showed approximately 26 per cent increased fruit set. Also the treatment applied during the middle of the pollination period was 20 per cent more effective than at other times. Variations occurred with different distances of the flowers from the tip of the shoot. The experimental results were said to be of a preliminary nature but suggested the possibility that someday the fig growers may spray their trees with plant growth regulators to take the place of pollination, thereby eliminating the necessity for using disease-carrying fig wasps.

With unpublished data the present reviewers (50) showed that *Vanilla* beans (seed pods) could be set by the application of indolebutyric acid (4 mg. per ml. 50 per cent alcohol) in alcoholic solution to take the place of hand pollination. The solution was applied with a camel's hair brush to the pistil of the flower. The beans which developed (95 per cent of treated flowers) appeared like normal fruit but without seeds. The value of these parthenocarpic fruit for vanillin content has not been tested.

Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on progeny seedlings.—Since plants are greatly modified in growth after treatment with physiologically active substances, the question has frequently been raised whether these effects carry over into the second generation. Pridham (51) found that seedlings of red kidney bean from parents sprayed with 2,4-D during the ripening of the pods showed

a range of 2,4-D symptoms in the juvenile and mature foliage. Virus-like crisp dwarfing, serration, and fusion of leaflets occurred to some degree in all seedlings. It is not entirely clear whether the holdover effects are due to substances adhering to the surface of the bean seed or whether the chemical is actually stored in the embryo. This can be tested by applying the chemical to the soil rather than using it as a spray. During the ripening period it could be assumed that the chemical would be transported up the stem and stored in the developing embryo.

Hitchcock & Zimmerman (52) found that seed from dandelions sprayed with 0.1 per cent 2,4,6-trichlorophenoxyacetic acid was delayed in germinating and gave a lower percentage germination as compared with seed from control plants. Later unpublished results showed that 2,4-D produced the same effects when used at a concentration of 0.001 per cent. In addition to the results described for dandelion, progeny from treated Patience dock exhibited similar abnormal effects. The progeny seedlings from dock plants, treated with 0.1 per cent 2,4-D before the seed was fully mature, were delayed in germinating and in early seedling growth and produced abnormally small modified leaves.

Willard (53) has recently reported a similar instance of abnormal effects being transmitted through the seed of corn grown in single-cross fields. In this case seed from plants treated with 2,4-D before tasseling was of low germination and the progeny was abnormal, slow growing, and low yielding. The greatest 2,4-D effects occurred in progeny from treated plants showing the least amount of injury in the field.

HORMONE WEED CONTROL

Since 1945 the use of 2,4-dichlorophenoxyacetic acid (2,4-D) for controlling weeds has developed so rapidly that any current evaluation of weed control is out of date by the time the report is published. It is not expected that the present review will be an exception. An account of the work which led to the use of 2,4-D as a weed killer has been described in several recent reports (4, 5, 54), which are concerned mainly with work on weed control prior to 1947 or with other uses of 2,4-D. The present review deals primarily with reports of weed control by means of 2,4-D and other hormone types of weed killers which were available between January 1947 and February 1948.

The most comprehensive account of weed control carried out during 1947 is contained in the Report of the Research Committee of the North Central Weed Control Conference issued in December 1947 (55), which consists of fifteen sections, each with a topical title. Individual abstracts are cited by the topical titles in the Literature Cited. This report describes the results of some two hundred experiments carried out by eighty investigators located in twenty-four states in this country and in six Canadian provinces. A summary of all results appears in the form of recommendations for the use of 2,4-D and other herbicides in the Report of the Policy Committee (71). Results in the area covered by this report appear to be representative of the results obtained elsewhere, mainly because the manufacturers of herbicides and of equipment for their application have cooperated with research groups throughout the nation.

Grains, flax, and sugar cane.—From the standpoint of economic importance, weed control in small grains, flax, and corn heads the list of crops. In order to eradicate noxious weeds commonly associated with these crops, it was found that the amounts of 2,4-D required were considerably in excess of those tolerated by the crops [68 (a)]. With dosages of 2 to 4 lbs. per acre, such weeds as Canada thistle, field bindweed, hoary cress, leafy spurge, and Russian knapweed could not be eradicated even in noncrop areas (56 to 62, 64) whereas 0.12 to 0.5 lb. per acre was the dosage range considered safe for spring grains and flax [63, 68 (a)]. Obviously, weed control in this case becomes a matter of minimizing the adverse effects of weeds, with dosages of 2,4-D which will not produce undesirable effects on the crop, rather than attempting to eradicate weeds or cause a high percentage kill. Only when the weed hazard outweighs slight losses in yield would the use of amounts of 2,4-D in excess of 0.5 lb. per acre on the above-mentioned crops be justified, as for example, to facilitate harvesting of areas which otherwise might be abandoned (71). Even in the latter case, such areas might be handled by spot treatment, particularly by means of low volume application (53).

Recommendations for treatment of spring grains and flax depend, as also in the case of other crops, upon a number of factors which appear to influence the effectiveness of 2,4-D both on weeds and crop plants. Among these are stage of development, rate of growth, soil and atmospheric conditions, and the variety or species

of plant [63, 68 (b), 70 (a), 71]. For the north central states and Canada 0.25 lb. per acre is considered the maximum safe dose for controlling broad-leaved annuals, being applied at full tiller stage on spring grains and to flax when the plants are about four inches high or in the rosette stage (63, 71). Some varieties may tolerate higher dosages [68 (b), 70 (b), (c), 71]. To obtain control of perennial weeds, amounts up to 0.75 lb. per acre are required and should be applied at late tiller or early shooting stage on spring grains, and on flax when the plants are six inches high or in the first bud stage but before weeds bloom (63, 71). Amounts in excess of 0.25 lb. per acre are likely to reduce the yield of flax and hence should be used with caution (63). Amounts of 0.25 to 1.0 lb. per acre are considered safe for winter wheat if applied when fully tillered or after bloom (71).

It is generally agreed that esters of 2,4-D are more hazardous to crops than the amide or sodium salt so that the esters should in general be used at the minimum recommended rates (63, 71). For spring grains and flax in Canada, the recommended rates for esters are about one-half of those for sodium salts (72). Under optimal conditions with respect to weeds and crop there has been little or no consistent difference in the relative effectiveness of different formulations, but under suboptimal conditions the esters have been noticeably more effective on weeds and more injurious to crops as compared with the salts (71). When legumes are grown as companion crops in small grains, 2,4-D cannot be used because of the greater sensitivity of legumes (73). Nonhormone herbicides are used in this case (63, 71).

Treatment of corn with 2,4-D sprays has given variable results, but they have been sufficiently promising to warrant further testing [68 (c), (d), (e), (f), 70 (a)]. Post-emergence treatments not exceeding 0.5 lb. per acre are not likely to cause the adverse effects resulting from higher rates, namely, stem bending, stalk brittleness, leaf roll which sometimes prevents tassel emergence, abnormal prop roots, and stunting, although rates up to 1 lb. per acre have not reduced yields significantly [68 (d)]. One case is reported of weak stems near soil level which was associated with abnormal anchor roots [69(c)] and in another case the anchor roots of sweet corn were strengthened by 2,4-D pre-emergence treatment [69 (b)]. A specific warning is given not to treat single-cross fields of corn in Ohio because of likely injury to pure line plants and to seed when 2,4-D is applied before tasseling (53). Less injury occurred when

the 2,4-D spray was directed away from the tops of the plants (53, 74). In California ground and airplane spraying at the rate of 1.25 lb. per acre controlled weeds without causing injury to field corn (75). Post-emergence treatments have controlled most broad-leaved annuals and perennials, but not weedy grasses. This is in contrast to pre-emergence treatments which control broad-leaved annuals and weedy grasses, but not broad-leaved perennials (53). Hybrid corn has shown considerable difference in tolerance to treatment with 2,4-D [68 (e), 70 (a), (d)].

Pre-emergence treatment has aroused considerable interest because it is the only type of treatment thus far developed which controls both broad-leaved annuals and weedy grasses such as crabgrass and foxtail (53). In view of the higher dosages applied to corn (1.5 to 5 lb. per acre) it has been suggested that the cost may be reduced by treating only over the corn rows, leaving the middles to be handled by cultivation (53). 2,4-D has been applied immediately after planting and also at later periods up to two days before seedling emergence. The recommendation for Ohio is to apply 2,4-D by any method immediately after planting (53), in which case weed control lasts for at least four weeks. In New Jersey applications made about a week after planting are preferred (76).

Rice weeds such as indigo, curly indigo, Mexican weed, and alligator weed have been controlled with 0.1 per cent 2,4-D sprays applied at one hundred gallons per acre on dry foliage and with 10 to 15 per cent 2,4-D dusts applied at the rate of 10 to 15 lb. per acre three days before, or about two weeks after flooding and when the foliage is wet with dew (77, 78, 79). Dusting is preferred because of the lighter equipment used and the favorable results obtained, dusts being equal to or better than sprays.

Sugar cane weeds, particularly alligator weed, were controlled by 0.2 per cent 2,4-D sprays applied at the rate of one hundred gallons per acre after the dew had dried on the foliage (77, 80). Proper integration of all parts of the spray equipment with speed of tractor is emphasized. This, of course, applies to spraying of other crops. Dusting was equally effective but it is not recommended if cotton is grown nearby (77). There was no adverse influence of the treatments on the sucrose content and purity under conditions which increased yields.

Vegetable crops.—Wide scale use of 2,4-D on vegetables during 1947 gave variable results because relatively little was known about important limiting factors. Sufficiently favorable results

were obtained to warrant the belief that 2,4-D may supplant the use of nonhormone herbicides on certain crops, particularly with respect to pre-emergence treatments. Pre-emergence treatments of asparagus, onion, beet, carrot, squash, tomato, and potato gave more favorable results than were obtained with other vegetable crops (67). It is not certain with which crops preplanting or post-planting treatments should be used. Preplanting applications were less effective for weed control on potato and onion and were more injurious to onion than postplanting applications (67). The methyl ester of α -naphthaleneacetic acid was equal to 2,4-D for controlling weeds by pre-emergence treatments, but the effects on yield of bean and corn were not given (81).

In tests with twenty-five vegetable crops pre-emergence treatment with 1.32 lb. of 2,4-D butyl ester per acre, applied one day after planting, controlled weeds for six weeks without causing noticeable injury to sweet corn, snap bean, mung bean, potato, and asparagus [69 (e)]. When applied immediately after planting, 0.66 lb. per acre controlled weeds for three weeks without noticeable injury to the crops just mentioned and also cucumber, lima bean, pea, and tomato. The most sensitive crops were *Brassica* and lettuce. Asparagus has been treated successfully at the end of the cutting season with 1.25 lb. of 2,4-D sodium salt per acre [69 (a)]. Pre-emergence treatment with 2,4-D sodium salt at about 3 lb. per acre (dry form with sand) gave favorable results with spinach, beet, onion, and carrot when applications were made nine days after planting [69 (f)].

Post-emergence treatment of strawberry with about 0.1 per cent 2,4-D has given favorable results although varietal differences were noted [69 (d), 70 (e)]. Presumably strawberry is reasonably tolerant except in flowering or fruiting stages [69 (d), 82, 83].

Lawns and turf areas.—Relatively few fundamental changes have occurred in the procedures for treating lawns and other turf areas, and there is still no exact agreement as to which weeds are readily eradicated by 2,4-D (4, 5, 55, 72, 73, 84, 85, 86). The tendency is toward the use of dry preparations of 2,4-D in combination with fertilizer, making use of a fertilizer spreader which probably represents the simplest and most efficient method of applying 2,4-D on the average sized lawn. In one large scale test involving several thousand treated plots, there was little difference in the effectiveness of dry forms of 2,4-D (3 to 8 lb. per acre) for preventing regrowth of dandelion, narrow- and broad-leaved plan-

tains, and white clover (87). There was likewise little or no difference in the formulations. Regrowth was appreciable in nearly all treated plots the same year and the following year, but no attempt was made to determine how much was due to reinfestation. Dry applications were considered fully as effective as sprays when used at about twice the rate, that is, 2 to 3 lb. per acre.

Where all dandelions have been counted before and after treatment, it has been shown that the roots are not always killed with a standard treatment of 2,4-D, even in cases where the top kill is 100 per cent (88). In these latter tests the highest percentage regrowth occurred in some cases in plots where the most rapid top kill had been obtained. It is not certain, in such special cases when very rapid killing of tops occurs, that a maximum transport of 2,4-D is prevented. In the case of bindweed a sufficient amount of 2,4-D had moved to the roots in twenty-four hours to retard shoot growth from severed pieces of rhizome, and within nine days maximum transport of 2,4-D had occurred, although all tops were not killed until twenty-one days after treatment (65). The general recommendations are still to use minimum amounts of 2,4-D on lawn grasses, particularly if bent, including redtop, is present (4, 5, 71, 73).

Woody plants.—Control of woody plants involves many problems not encountered in the control of herbaceous weeds. Most interest has been centered in the control of brush which varies more in height and involves more resistant species than in the case of field crops. Good coverage is essential, but it is not always easy to attain. Low volume spraying may prove effective, but the minimum volumes are likely to be in the range ten to twenty gallons rather than five gallons or less (89). Many important limiting factors in brush control were described at the Northeastern Weed Control Conference held in New York City February 12-13, 1948 (90, 91) and also by others (66, 89). Higher rates of application have always been used in brush control than in the control of herbaceous weeds, and it is frequently necessary to combine mechanical methods with spray operations. In one case effective killing was considered due primarily to the characteristics of the spray solution, for which all active ingredients were not divulged (90). If chlorinated phenols in combination with 2,4-D should prove as effective on brush as on tropical woody plants (92, 93), the problem of brush control might be simplified. The increased use of 2,4,5-trichlorophenoxyacetic acid alone and in combination with

2,4-D for killing brush is expected during 1948 (91). On some herbaceous plants 2,4,5-trichlorophenoxyacetic compounds have been less effective than 2,4-D (27, 94).

Rights of way frequently run through forests and other areas where precautions must be taken to avoid injury to other plants. It has been shown that seedling pines are injured but that older plants are more resistant (95). Safe use of 2,4-D in nursery plantings can scarcely be recommended until more information is available. The use of high rates of 2,4-D on plowed soil has been suggested (96), but it seems unlikely that amounts up to 37 lb. per acre are justified.

Action of 2,4-dichlorophenoxyacetic acid (2,4-D).—The one thing which all plant hormones have in common is the capacity to be transported from the region of application to other parts of the plant where specific types of growth responses are induced. Transport has been shown to occur mainly in the xylem when introduced into the transpiration stream through the roots or through cut surfaces of aerial parts, and that upward or downward movement is limited by conditions which influence transpiration, but that a dead segment of stem does not prevent movement in either direction (97). In contrast, it was shown that movement was mainly outside the xylem when the hormone was applied to intact aerial parts, and in this case there was little or no movement through a dead segment of stem. Notwithstanding the extensive information on hormone transport, there are only a few cases in which the applied hormone has been detected or identified by direct methods beyond the region of application, namely, by a modified Winkler indole test and by x-ray analysis (98). However, up to 1947 there was still lacking substantial quantitative data relating to the exact amounts of hormone transported and to the paths of transport outside the transpiration stream. It is of special interest, therefore, that recent reports appearing in 1947 furnish additional information on these two points (28, 29, 99, 100, 101).

The transport of 2,4-D has been shown to be associated with the translocation of organic food reserves to the extent that movement of both from leaves to stem does not occur under the special conditions when there is no photosynthetic activity and when the food reserves are depleted (99). To what extent these conditions limit the herbicidal activity of 2,4-D in the field has not yet been demonstrated. The suggestion that the stimulated metabolism caused by 2,4-D somehow finally causes interference in phloem

function (100) would appear to postulate a transport of 2,4-D independently of reserve food movement. The lesser effectiveness of 2,4-D on poison ivy and also other plants (102) growing in the shade may be explainable on the basis of inefficient 2,4-D transport resulting from low food reserves and a low level of photosynthetic activity which might be expected to occur under shade conditions. It is not clear how the same explanation would apply to Japanese honeysuckle, which is killed most readily under shade conditions (103).

The translocation of radioactive 2-iodo¹³¹-3-nitrobenzoic acid (INBA) in bean and barley (29) furnishes quantitative data on the amounts of the applied hormone which are translocated to and accumulated in different parts of the plant. When INBA was applied near the tip of one leaf of the first pair on bean seedlings, movement was mainly to meristematic regions where growth was inhibited, but only a trace was found in the opposite leaf. The quantities of INBA were determined by a Geiger counter on isolated segments of the plant and the path of movement was shown photographically. Presumably the INBA was translocated in the undissociated form, but conclusive proof of this fact has not yet been furnished. In contrast, the movement of INBA from near the tip of the first leaf of barley seedlings was mainly to the second leaf where the greatest accumulation occurred without inhibiting the growth of the leaf. The accumulation of INBA decreased toward the base of the seedling, none being found in the roots. This may help to explain why perennial lawn grasses frequently show rapid recovery after the tops have been damaged by toxic dosages of 2,4-D, if it can be shown that 2,4-D, like INBA, is not transported to the roots of grasses.

The inhibitive effects of INBA on growing points in the bean are similar to those described for 2,3,5-triiodobenzoic acid on tomato whereby vegetative growing points were inhibited and the accumulative effects at the nodes induced slight nodal bending which resulted in the production of zigzag shaped stems (104). In addition to these responses, 2,3,5-triiodobenzoic acid completely disrupted the flowering habits of the tomato, causing the plant to produce flower shoots of abnormal character as growing points. Since the effects just described are induced only by the substituted benzoic acid compounds, it remains to be seen whether the results obtained with INBA or other substituted benzoic acids can serve as a basis for explaining the action of 2,4-D or other classes of

hormones. Were it not for the relatively high cost and unavailability of the substituted benzoic acids, they might be used to advantage in combination with 2,4-D for killing plants or inhibiting their growth.

Abnormal characteristics with respect to germination, growth, and yield have been transmitted to progeny seedlings of bean (51), dandelion and dock (88), and corn (53). In all cases the 2,4-D treatments were applied to the parent plants before the seed had matured. A similar effect has been reported for buds on fruit trees which were delayed in opening in the spring as a result of hormone treatment applied during the previous summer and autumn when the buds were immature (36). Excessive doses will cause extreme types of distorted foliage (105). Such severe damage occurred to parent corn plants and to progeny seedlings in the case of pure line strains that farmers in Ohio are warned not to use 2,4-D on single-cross fields (53).

New developments in weed control.—One of the outstanding contributions during 1947 was the development of equipment for low volume spraying whereby five gallons or less of a spray solution can be dispersed evenly on one acre of land (53, 106). The need for using such small volumes has been recognized for some time, particularly for regions where water is scarce and, as in rice fields, where the use of heavy equipment designed for dilute spraying is not always feasible. In some cases existing equipment has been modified to disperse sprays at the rate of ten to fifty gallons per acre (75, 107). Limited experience with low volume spraying in the range of five gallons per acre or less has indicated that complete or uniform coverage may not be obtained under all conditions, so that with present spray equipment and stock herbicides, volumes in the range of five to ten gallons per acre are likely to give more consistent results than lower volumes (53, 90).

Pre-emergence treatment, whereby the herbicide is applied any time before emergence of the crop, is regarded as one of the most important relatively recent developments in weed control. Although variable in results (67, 71), the pre-emergence treatment of vegetable crops is probably the most likely method for future use and perhaps the only method which can be used safely on this relatively sensitive class of crops. Pre-emergence treatment of corn may prove of sufficient importance to change the method of planting from check row to drilling (53). Considering both pre-emergence and post-emergence treatments, it has been suggested that

2,4-D may prove of greater importance to the farmer than the introduction of hybrid corn (53). Results with gladiolus (108) indicate that pre-emergence treatment may be used to advantage if planting of corms is delayed for two to three weeks.

The possibility of increasing the herbicidal effectiveness of 2,4-D by means of activators or adjuvants has received little attention except in the case of carriers where emphasis has been placed on solubilizing characteristics particularly as applied to the esters of 2,4-D. There are only a few reports to date which deal with tests designed to show activation of 2,4-D by means of other hormones or by nonhormone compounds. Certain indole, naphthalene, and phenyl compounds and those containing various forms of nitrogen, potassium, and phosphorus were among the first used for increasing the herbicidal activity of 2,4-D, in this case on water hyacinth (109). This type of activation is similar to that reported for root induction in cuttings, in which case the above mentioned hormones, and also certain vitamins, increased the root-inducing activity of indolebutyric acid, α -naphthaleneacetic acid, or 2,4-D (110, 111).

Since activation of 2,4-D did not depend upon structural specificity, the next logical step was the use of highly toxic activators such as arsenicals, chlorates, sulfamates, and chlorinated phenols, all of which have been used as contact herbicides. When relatively small amounts of these toxicants were combined with 2,4-D in spray mixtures, the herbicidal activity of 2,4-D was greatly enhanced (54, 84, 92, 93, 112). The statement that these results were not to be expected on the basis of previous work on translocation of 2,4-D (54, 93) is surprising, since in the work referred to (99) there were no data to show how toxicants might limit the transport of 2,4-D. Another example of an effective 2,4-D mixture is that which resulted in a complete killing of woody plants, including white ash, but the identity of the activators or adjuvants was not disclosed (90). Mixtures of α -naphthaleneacetic acid and 2,4-D were more effective in killing the roots of dandelion than either of the individual components (88). In pre-emergence tests, methyl- α -naphthaleneacetate (7.5 lb. per acre) was equal to 2,4-D in controlling weeds (81). It has been suggested (91) that mixtures of 2,4-D and 2,4,5-trichlorophenoxyacetic acid should be more effective, particularly on woody plants, than 2,4-D alone, on the basis that 2,4,5-trichlorophenoxyacetic acid has proven more effective than 2,4-D for killing shrubs and trees (91, 94) and certain species

of *Rubus* (89, 91). The higher root-inducing activity of 2,4,5-trichlorophenoxy acids as compared with 2,4-D has also been reported (113).

In some recent experiments (unpublished) the reviewers found 2,4-D mixtures containing Benoclor 3C (114), sulfamate, or arsenicals to be more effective in killing tomato plants than either component alone. These mixtures were also more effective in killing volunteer seedlings of crabgrass, smartweed, purslane, and chickweed. Solutions containing 1 to 3 per cent Benoclor 3C and 0.05 to 0.1 per cent 2,4-D were nearly as effective when applied successively to tomato, after an interval of twenty-four hours, as when applied as mixtures. Essentially the same results were obtained regardless of which component was applied first. Furthermore, the hormone-bending responses induced by the mixtures were much more pronounced than those induced by 2,4-D, notwithstanding that the activator in this case was a nonhormone compound which does not itself cause bending. Similar activation of 2,4-D was obtained with 2,3,5-triiodobenzoic acid.

Presumably nonhormone adjuvants like Benoclor 3C alter the permeability of the tissue so as to aid the penetration and possibly also the transport of 2,4-D. Differences in 2,4-D formulations are probably explainable to a large extent upon the action of carrier ingredients which bring about better penetration of 2,4-D. Undiluted Benoclor 3C concentrate is used for the control of aquatic weeds in irrigation ditches and canals (115). Being heavier than water (sp. gr. 1.4) this material settles gradually over the submerged plants after being dispersed with pressure a few inches under the surface of the water. It is believed that 2,4-D might be used to advantage with Benoclor 3C either in mixtures or as successive treatments for killing submerged, partly emergent, or floating plants. There is relatively little published information on the control of aquatic and marginal water plants by means of hormone weed killers. Recent extensive tests with 2,4-D carried out along the waterways of the Tennessee Valley Authority indicated marked variation in killing under what appeared to be similar conditions (116). Considering the importance of aquatic weed control even apart from its value in conjunction with mosquito control in the South, it seems likely that this phase of weed control will eventually show progress as in the case of crop plants.

Sodium isopropyl xanthate and allyl mixed chlorophenyl carbonate were reported as new herbicides (117). The first named is

of the complete killer type and may have its main use in pre-emergence treatments. The second named is classed as a selective weed killer, particularly useful against weedy grasses, and is said to possess hormone-like properties.

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BACTERIAL METABOLISM¹

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This review will be limited largely to papers which emphasize the chemical aspects of bacterial metabolism and which have appeared during the past year. However, there is bound to be a certain amount of overlapping with enzyme studies and with growth factor and nutrition studies in relation to metabolic function. Because of space limitation and since adequate articles on growth factors are available, this phase of metabolism will be omitted unless the factors concerned deal specifically with metabolic function. Also omitted is any consideration of antibiotics and related metabolic products unless they deal with enzymes directly.

The past year has witnessed the continued development of several lines of research as well as the advent of a few new ones. Perhaps of major interest, as indicated both by the number and quality of contributions, are the studies on the function of essential metabolites. Highly significant additions have also been made to the knowledge of enzyme mechanisms, specifically in the enzyme-substrate studies with transglucosidase (1) and in studies of the mechanism of amino acid formation and degradation. On the problem of amino acid assimilation, the studies of Gale and his group (2) and of Hotchkiss (3) should be noted. In addition, the mechanism of biological protein synthesis, a problem not unrelated to the assimilation of amino acids by bacterial cells, has received attention and promising advances have been made. Microbiological studies are also contributing much to the knowledge of the mechanism of enzyme formation, especially with regard to adaptive enzymes (4).

In addition to the enzyme methods for approaching the synthetic and degradative processes of microorganisms, the competitive inhibitor method, used so successfully by Shive & Macow (5) and others to predict the function of essential metabolites, should

¹ This review covers the period from November 1946 to December 1947.

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be considered. The studies of Lampen & Jones (6) present further evidence as to the sequence of reactions of metabolic processes.

A most significant trend is to be noted in the number of bacteriologists and biochemists who are turning to bacterial enzymes as means of studying fundamental biochemical problems. As this year closes, one has the feeling that an approach is being made to the metabolism of amino acids, their mode of synthesis, and the synthesis of proteins in a fashion not unlike that which characterized the study of carbohydrate metabolism a decade or less ago.

It is assumed that the reader is familiar with the new *Annual Review of Microbiology* as well as *Advances in Enzymology*, *Vitamins and Hormones*, *Carbohydrate Chemistry*, and *Proteins and Enzymes*, which contain reviews on specific phases of microbial metabolism. A symposium on heredity and variation in microorganisms [*Cold Spring Harbor Symposium*, 11 (1946)] includes reports of metabolic studies involving mutants of *Neurospora* (7) and bacteria (8), as well as articles on the influence of environment on metabolism (9, 10) and of the factors controlling enzyme formation (4).

Kluyver & Custers (11) have abstracted the papers on bacterial metabolism which appeared in the Netherlands journals during the war years. A series of papers in honor of Dr. Kluyver's twenty-fifth year as professor of microbiology at Delft by his students and colleagues appears in Volume 12 of *Antonie van Leeuwenhoek Journal of Microbiology and Serology* (12). A book on chemical activities of bacteria by Gale (13), not as yet available to the author, has just appeared.

OXIDATION

Several new aids to respiration studies have been suggested. Dorrell & Knight (14) have performed respiration studies with fungus homogenates. Endogenous respiration, which is usually high with mold preparations, may be decreased by aging the homogenates at a low temperature or by growing the cultures on carbohydrate-poor media. Stanier (15) suggested a method for determining the route of oxidation of various substrates based on multiple simultaneous adaptation and has employed the method to study the oxidation of a series of aromatic compounds. According to this hypothesis, cells grown on a given substrate should attack all intermediate compounds in the reaction series, whereas growth on a compound intermediate in the series should cause

adaptation to all subsequent members of the series. With the compounds studied, resting cells could also be adapted by incubation with the substrates for periods of twenty to sixty minutes.

Keilin & Hartree (16) have studied the cytochrome content of vegetative cells and spores of *Bacillus subtilis*. The absorption bands for cytochromes-*a*, -*b*, and -*c* were present in vegetative cells, and their respiration was sensitive to the usual inhibitors for iron systems. The spores exhibited slight respiration and showed only about 6 per cent as much cytochrome as the vegetative cells; the spores do, however, contain about 50 per cent as much hematin as the vegetative cells. The suggestion was made that this may serve as a precursor of the cytochromes.

Dole, Hawkins & Barker (17) have studied the fractionation of oxygen isotopes by growing bacterial cultures and found a very slight preferential use of oxygen 16 over oxygen 18. Whelton & Phaff (18) prepared a stable nonoxidative variant of *Saccharomyces cerevisiae* by the use of ethylene oxide.

Bellamy & Klimek (19) have reported the metabolic characteristics of a penicillin-resistant strain of *Staphylococcus aureus*. A strain with a sixty thousandfold increase in penicillin resistance was gram negative and grew only aerobically, also more slowly than the parent strain. Organisms such as *Streptococcus faecalis* and *Clostridium perfringens* which lack aerobic mechanisms did not develop penicillin resistance; this led to the suggestion that ability to develop penicillin resistance is associated with aerobiosis. [For the effect of penicillin on assimilation, see Gale (113).]

Organic acid oxidation.—Lominski *et al.* (20) studied the utilization of citric acid by *Escherichia coli*, which is not generally considered to be a citrate-utilizing organism. If the organism were grown in a peptone-citrate medium, adaptation to citrate oxidation occurred. *Staphylococcus aureus* was found not to use this substrate.

Kalnitsky & Barron (21) utilized fluoroacetate as an inhibitor of oxidative processes in various organisms, among them *Escherichia coli* and *Corynebacterium creatinovorans*; with the latter organism fluoroacetate, at a concentration of 0.002 *M*, was a very effective inhibitor of acetate, succinate, or malate oxidation. The oxidation of other compounds structurally similar to fluoroacetate such as glycine, glycollic, and glyoxalic acids was also markedly inhibited. Pyruvate oxidation by *Escherichia coli* and the gonococcus were inhibited to a smaller extent. Fluoroacetate is considered

to compete for enzyme with acetate and related compounds because of the similar bond distances of fluorine and hydrogen. Randles & Birkeland (22) reported that malonate inhibits methylene blue reduction with succinate as substrate by *Pseudomonas aeruginosa* but not by *Escherichia coli*. If *E. coli* was grown under aerobic conditions in the presence of acetate, the rate of methylene blue reduction with a series of organic acids, including acetate, succinate, fumarate, and malate, was greatly increased, thus indicating that these may mediate in the oxidation of acetate by this organism.

Lwoff *et al.* (23, 24) have presented evidence that a mutant of *Moraxella lwoffii* oxidized succinate, fumarate, and malate by a mechanism independent of the usual route through oxaloacetate followed by decarboxylation to pyruvate and carbon dioxide. It is suggested that pyruvate may be formed directly from malate without oxaloacetate serving as an intermediate. The triphosphopyridine nucleotide-linked malic acid decarboxylase of pigeon liver described by Ochoa *et al.* (25) answers this description. Ajl, White & Werkman (26) have shown that a number of C₄ dicarboxylic acids can fulfill the carbon dioxide requirement of heterotrophic organisms especially *Aerobacter aerogenes* and *Escherichia coli*, thus indicating the mediation of these compounds in the metabolism of the organisms mentioned.

The oxidation processes of a saprophytic acid fast organism, *Mycobacterium phlei*, has been explored by Edson & Hunter (27). With resting cells a series of carbohydrates and organic acids was found to undergo oxidation through a cyanide-sensitive series of carriers. A stable lactate enzyme was obtained in acetone powders and was found to catalyze the oxidation as far as acetate and carbon dioxide (28). This enzyme is noncyanide sensitive and flavine-adenine dinucleotide linked. The oxidation of hydroxy acids by several acid fast strains was investigated by Roulet *et al.* (29).

Carbohydrates and alcohols.—The number of oxidative reactions catalyzed by members of the genus *Acetobacter* and the genus *Pseudomonas* has been extended. Stanier (30, 31) has shown that ethyl alcohol is oxidized by *Pseudomonas fluorescens* to yield as much as 50 to 70 per cent of the calculated amount of acetic acid, thus adding acetic acid formation to the series of similarities between the *Acetobacter* and *Pseudomonas* genera. *Pseudomonas*

strains are in general relatively more acid sensitive. The oxidation of pentoses as far as the corresponding *onic* acids by several *Pseudomonas* strains has been reported by Lockwood & Nelson (32).

Carter *et al.* (33) found the oxidation of *meso*-inositol by *Acetobacter suboxidans* to yield scyllo-*meso*-inosose, and upon further oxidation, a product which was not isolated, possibly di-keto-inositol.

Mickelson & Shideman (34) studied the oxidation of glycerol by *Escherichia coli*. Inorganic phosphate disappeared with the probable formation of glycerol phosphate, since α -glycerol phosphate was also oxidized. Both ATP and inorganic phosphate stimulated the rate of oxidation. The respiration is both cyanide and iodoacetate sensitive, therefore, resembling the yeast enzyme system.

Reese (35) has studied the conditions necessary for the growth of cellulose-decomposing bacteria and has shown that oxidation is necessary for growth of the organisms. The enhancement of oxidation by the addition of iron indicates the presence of typical aerobic mechanisms.

Aromatic compounds.—The oxidation of a series of aromatic compounds by *Pseudomonas fluorescens* has been studied by Stanier (15) with his multiple simultaneous adaptation method. Mandelic and benzoic acid oxidation appears to occur through a similar series of enzymes; the other acids tested, phenylacetic and *p*-hydroxybenzoic acid, did not adapt during the oxidation of the former and, therefore, do not appear to be intermediates in the oxidation chain. From the curves presented, it appears that these compounds undergo approximately one-third complete oxidation. Investigation of the cause for the later reaction would be interesting to investigate, as would the other intermediates in the oxidation.

The oxidation of phenol and benzoic acid by a series of soil organisms has been shown by Evans (36) to occur through the analogous dihydroxy compounds followed by ring cleavage. Complete oxidation did not occur since only four moles of carbon dioxide were released per mole of phenol oxidized. From the data one cannot differentiate between assimilation and the accumulation of intermediate products as the cause of the incomplete oxidation.

The oxidation of carcinogenic hydrocarbons by marine bacteria was reported by Sisler & ZoBell (37), but precise data are not available as yet.

Hydrogenases.—Stephenson (38) has reviewed several phases of the hydrogen transport problem, especially with relation to the hydrogenases. The hydrogenases of *Proteus vulgaris* have been studied by Farkas & Fischer (39) who determined the characteristics of the enzymes by the exchange reaction between hydrogen and deuterium as well as by the reduction of fumarate. Fumarate reduction with hydrogen was also studied with *Escherichia coli* (40). It was concluded that two enzymes exist—one for hydrogen activation, a second for the activation of fumarate. Back and co-workers (41) studied the hydrogenase of *Escherichia coli* with cell free extracts and found oxygen, methylene blue, fumarate, and nitrate to serve as suitable hydrogen acceptors.

Lascelles & Still (42) have studied the nitrate, nitrite, and hydroxylamine reduction systems of *Escherichia coli* in the presence of hydrogen and have concluded from the optimum pH, effect of inhibitors, etc., that the three are quite distinct. Pollock (43) studied the adaptive nature of the nitratase of *E. coli* and the influence of various hydrogen donors upon this system.

Aubel *et al.* (44) determined the effect of azide on the anaerobic evolution of hydrogen by *Escherichia coli* and found that 0.0005 *M* would inhibit hydrogen release from either glucose or pyruvate, whereas 0.001 *M* did not inhibit respiration on these substrates.

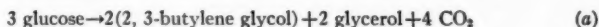
Oxidation by sulfate-reducing bacteria.—The oxidation of hydrocarbons (45) and even numbered carbon fatty acids (46) by the sulfate reducing bacteria has been reported by Rosenfeld. In the presence of reduced methylene blue as hydrogen donor, fatty acids were reduced by this organism. Butlin & Adams (47) have demonstrated that the sulfate-reducing organism, *Vibrio desulfuricans*, can grow autotrophically in the presence of hydrogen as an energy source.

FERMENTATION

Meyerhof (48) has reviewed his recent studies on the kinetics of cell-free alcoholic fermentation. These include the mechanism of the arsenate effect, the evidence for the existence of a 1,3-diphospho glyceraldehyde complex, and the relationship of adenosinetri-

phosphatase to the Harden-Young equation for hexosediphosphate accumulation.

Bacilli.—The study of the butylene glycol fermentation of aerobic bacilli which was begun during the war has been continued. *Bacillus subtilis*, both the Lawrence & Ford and the Marburg types, produce good yields of 2,3-butylen glycol, acetylmethylcarbinol, and glycerol aerobically (49). The former also produced solvents anaerobically, but the Marburg type did not grow under these conditions. The following equation has been suggested to describe the route of 80 to 90 per cent of the glucose fermented with selected strains.



With many strains, however, the butylene glycol and glycerol yields fell below these levels. Small amounts of formic, acetic, and lactic acids and traces of ethyl alcohol were also formed. The butylene glycol formed consists of about two thirds of the *meso* isomer and one third of the *laevo*, whereas the lactic acid formed is largely the *l* (+) isomer.

The influence of aeration and of potassium and nitrogen sources on butylene glycol production by *Bacillus polymyxa* has been studied (50, 51, 52). With aeration, the yield of butylene glycol is decreased with a corresponding increase in acetylmethylcarbinol formation; the sum of the two equalled about one-half mole per mole of glucose fermented (50).

Colon-aerogenes bacteria.—The fermentation products of *Serratia marcescens* (aerogenes type gram negative rod) include butylene glycol in yields as high as one-half mole per mole of glucose fermented (53), the other products being largely lactic and formic acids, ethyl alcohol and carbon dioxide. Aerobically, the yield of carbon dioxide is increased at the expense of these compounds without markedly affecting the yield of butylene glycol. As is usual for the gram negative rods, *d* (−) lactic acid is formed.

Factors affecting butylene glycol production by *Aerobacter aerogenes* have been studied by Freeman (54). In addition, Paretzky & Werkman (55) have shown aeration and methylene blue to favor the conversion of butylene glycol to acetylmethylcarbinol.

Genus Clostridium (butyric-butanol fermentation).—Simon (56) prepared resting cells and acetone-dried preparations of *Clos-*

tridium acetobutylicum with which he determined fermentability and the products from a series of substrates. Among the hexose sugars, sugar acids, and sugar esters, alteration of the first carbon did not affect the fermentation nor the yield of butyric acid, whereas alteration of the sixth position resulted either in nonfermentation or in a decreased yield of butyric acid. The fermentation of hexosediphosphate led to methyl glyoxal and pyruvate rather than to the normal products, whereas pyruvate fermentation proceeded only as far as acetate; i.e., it did not result in butyrate formation.

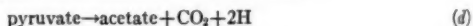
Further studies on fermentation in the presence of carbon monoxide (57) confirmed the formation of lactic acid as reported by Kempner (58). In addition, Simon showed that pyruvate breakdown was inhibited by carbon monoxide, thus suggesting that the pyruvate oxidase or an enzyme for a closely related subsequent step may be an iron-containing enzyme.

Barker (59) has discussed the characteristics of *Clostridium kluyveri* and the reactions which lead to the formation of the longer chain fatty acids with this culture.

Bhat & Barker (60) have isolated a new anaerobe, *Clostridium lacto-acetophilum*, capable of utilizing lactate as a carbon and energy source. Enrichment cultures grew well on lactate medium, but pure cultures required in addition the presence of acetate, biotin, *p*-aminobenzoic acid, and traces of yeast autolysate for growth. Lactate is broken down by the following suggested reactions:



The excess of acetate required in reaction (c) over reaction (a) indicates the function of acetate as an external hydrogen acceptor; otherwise, the excess of reduced product, hydrogen, stops the fermentation. During the fermentation of pyruvate,



fermentation proceeds without the addition of external hydrogen acceptor since the acetate and hydrogen are in the proportions required for reaction (c). Although growth with glucose as an energy source can occur quite normally with the release of carbon

dioxide and hydrogen, energy release from lactate apparently cannot occur without an external hydrogen acceptor. From the data the authors also suggest a relationship between the quantity of butyrate produced and the residual acetate; this may be conditioned by an equilibrium involving these compounds or may possibly be conditioned by the hydrogen pressure. The question whether other anaerobes may also utilize lactate as an energy source in the presence of suitable hydrogen acceptors is considered.

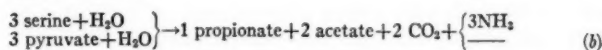
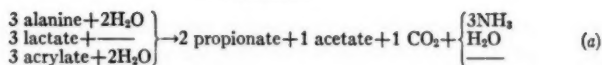
Barker & Elsdon (61) have reported the utilization of carbon dioxide in the formation of acetic acid and glycine by *Clostridium cylindrosporus*. Anaerobic fermentation of uric acid yields carbon dioxide, ammonia, glycine, and acetate. If the fermentation occurs in the presence of isotopically labeled carbon dioxide, the C^{14} appears in the methyl and carboxyl carbons of acetate and in the carboxyl carbon of glycine. Glycine and acetate appear to be formed by different mechanisms and are not interconvertible.

Aubel and co-workers (62) have studied the effect of oxygen on the oxidation-reduction potential and on the growth of cultures of the anaerobic organisms, *Clostridium saccharobutyricum* and *Clostridium sporogenes*. Aeration in the presence of a small number of cells prevented growth but not fermentation. Hydrogen peroxide (63) in low concentrations caused a reversible inhibition of fermentation; larger amounts stopped fermentation entirely.

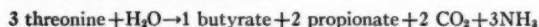
Tytell & Tytell (64) reported upon the conditions influencing glucose dehydrogenase production by *Clostridium perfringens*. Prévot & Enescu (65) studied nitrate reduction by this organism using methylene blue as a hydrogen acceptor. They also studied the fermentation products of *Clostridium corallinum* (66) and the diversion of the fermentation of *Clostridium bifermentans* and *Clostridium caproicum* (67).

Amino acid fermentation (propionic and acetic).—Two new amino acid-fermenting organisms have been isolated by Cardon & Barker (68, 69) using enrichment techniques. One of these, *Clostridium propionicum*, formed propionic acid from alanine and related compounds, whereas the second, *Diplococcus glycinophilus*, utilized only glycine as an energy source.

Fermentation balances, with resting cell suspensions of *Clostridium propionicum*, yielded two types of fermentation depending upon the state of oxidation of the substrates used. These may be written as follows:

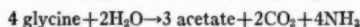


A third reaction, analogous to (b) except for the chain length of substrate, occurs with threonine:



A number of other amino acids are also attacked including glutamic, alanine, valine, leucine, phenylalanine, cystine, glycine, and, more slowly, histidine. This organism is not a true propionic acid bacterium but apparently carries out this type of fermentation in response to the oxidation-reduction state and the chain length of the acids attacked. It should also be noted that the fermentation of lactate by this organism differs from that which occurs with *Clostridium lacto-acetophilum* described in the previous section.

The second amino acid fermenting organism, *Diplococcus glycinophilus* (68, 69), utilizes only glycine as an energy source; this is broken down according to the following equation:



In the presence of glycine, pyruvate and serine are also broken down slowly. If fermentation occurs under conditions in which the gas pressure is relieved (decreased hydrogen pressure), hydrogen and carbon dioxide are produced with a decrease in acetate yield. The relationship of this amino acid fermentation to the reactions of other anaerobes in which glycine serves either as a hydrogen acceptor or donor is discussed.

Methane fermentation.—Kluyver & Schnellen (70) studied the fermentation of carbon monoxide by methane bacteria using *Methanosarcina barkeri* and reported the following reaction to occur:



This reaction has been shown to occur by route of the two following reactions:



Reaction (b) has been demonstrated by trapping the carbon dioxide with alkali, in which case, the calculated amount of hydrogen

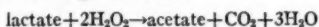
was found. Reaction (c) was demonstrated by methane production in the presence of hydrogen and carbon dioxide. In the presence of a hydrogen atmosphere, all the carbon monoxide may be converted to methane. *Methanobacterium omelianskii* does not utilize carbon monoxide and hydrogen; furthermore, in the presence of carbon dioxide, hydrogen, and carbon monoxide, methane is formed without carbon monoxide being used. Therefore, carbon monoxide cannot be an intermediate in the formation of methane from carbon dioxide.

Lactic acid bacteria and Staphylococci.—Two more reactions of pyruvate have been shown to be reversible. Wikén and co-workers (71) have shown by an exchange reaction with isotopic carbon dioxide that the dismutation reaction in this organism is reversible. Using a cell-free preparation in the presence of pyruvate, inorganic phosphate, and isotopically labeled carbon dioxide, the isotope was found in the carboxyl group of pyruvate. Thus it is suggested that the reaction, $2 \text{ pyruvate} + \text{phosphate} \rightleftharpoons 1 \text{ lactate} + 1 \text{ acetate (acetylphosphate)} + 1 \text{ CO}_2$, is reversible.

Watt & Krampitz (72) have also shown that isotopic carbon is fixed in the carboxyl group of pyruvate during acetylmethylcarbinol formation by *Staphylococcus aureus*. These workers have postulated the occurrence of α -acetolactic acid as an intermediate in this reaction because this acid is decarboxylated rapidly with the formation of acetylmethylcarbinol. Manganese but not cocarboxylase is required. If this were the mechanism, the carboxylation would occur in the beta position in analogy to other known beta carboxylases, as oxaloacetate, which require manganese but not cocarboxylase.

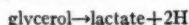
Gross & Werkman (73) confirmed the mechanism of acetylmethylcarbinol formation by *Aerobacter*, yeast and muscle tissue as occurring by three mechanisms, namely: *Aerobacter* forms acetylmethylcarbinol from two moles of pyruvate and does not utilize acetaldehyde, the yeast and pig heart enzymes involve one mole of pyruvate and one of acetaldehyde. The pig heart enzyme also forms acetylmethylcarbinol from acetaldehyde alone, apparently without endogenous pyruvate utilization, since no carbon dioxide is released. Using yeast juices (74) and isotopically labeled acetaldehyde, these workers found heavy carbon distributed throughout the acetylmethylcarbinol molecule and suggested the possibility of a symmetrical intermediate.

Douglas (75) found hydrogen peroxide to be utilized in the metabolism of *Lactobacillus brevis*. Lactic acid or glucose is oxidized to acetate plus carbon dioxide without the accumulation of peroxide. Furthermore, under anaerobic conditions, hydrogen peroxide served as a hydrogen acceptor for the oxidation of lactate according to the following reaction:

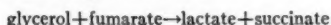


The cells did not activate lactate or peroxide alone nor did heat-killed cells bring about the destruction of peroxide, indicating that a peroxidase is involved. Douglas suggests that this enzyme is comparable to the noncyanide sensitive peroxidase enzyme of *Streptococcus mastitidis* previously reported by Greisen *et al.* (76).

Gunsalus (77) has reported the products of anaerobic glycerol fermentation by *Streptococcus faecalis*. In analogy to the reaction described by Bhat & Barker (60) for *Clostridium lacto-acetophilum*, this organism is unable to ferment glycerol in the absence of an external hydrogen acceptor. The general equation for the fermentation of glycerol by streptococci is:



The strain studied can utilize fumarate as a hydrogen acceptor according to the following reaction:



In the presence of excess fumarate, products more oxidized than lactate, i.e., acetate and carbon dioxide, are formed.

Hoff-Jørgensen, Williams & Snell (78) reported the preferential use of lactose by a strain of *Lactobacillus bulgaricus*. Their data were similar to those for the preferential utilization of disaccharides by other organisms (79, 80), except that this appears to constitute a limiting case in which monosaccharides are not utilized at all in the absence of the disaccharide.

Stimulation of the glycolytic rate of various lactic acid bacteria has been reported. In addition to McIlwain's (81) observation that glutamine stimulates the glycolytic rate, Foust & Gunsalus (82) have shown that glutamic acid, histidine, and ammonia will stimulate the rate of glycolysis of washed suspensions of *Streptococcus faecalis* as much as four- to fivefold. Woolley (83) has also reported that streptogenin will stimulate the glycolysis of washed cells of *Lactobacillus casei*. As yet the position or mode of action of these

effects is unknown. However, their relationship to the assimilation studies of Hotchkiss (3) and Gale (2) should be considered.

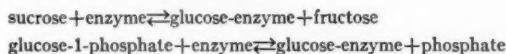
Tsuchiya & Halvorson (84) have reported upon the conditions necessary for the preparation of glycolytically active washed cells of lactobacilli; the resting cells could be lyophilized without great loss in activity. Halvorson & Muedeking (85) found greater fermentation rates of *Lactobacillus casei* on yeast extract media than on the best synthetic medium obtainable, but were unable to determine the cause for these differences.

BACTERIAL ENZYMES

Spiegelman (86) has discussed in some detail the factors involved in enzyme formation.

Disaccharide formation.—Several recent reviews on the formation of polysaccharides by microorganisms should be noted: Stacey (87) on bacterial cellulose and dextrans, capsular polysaccharides, and other macromolecules; Evans & Hibbert (88) on bacterial polysaccharide formation.

Doudoroff, Barker & Hassid (89), continuing their studies with partially purified sucrose phosphorylase of *Pseudomonas saccharophila*, have made very significant progress. They present evidence to indicate that, in the absence of phosphate, sorbose replaces fructose in the formation of glucosido-sorbose from sucrose; furthermore, glucose-1-phosphate undergoes an exchange reaction with isotopic phosphate in the absence of fructose. The indicated mechanisms may be written as follows:



Thus it appears that the enzyme can substitute a linkage for the analogous glucosido linkage in sucrose or in glucose-1-phosphate, thus adding substantial evidence to the enzyme substrate theory. These findings were interpreted as a glucose transport function, and the enzyme was renamed "transglucosidase." Two new disaccharides, one a nonreducing disaccharide and the second a reducing disaccharide have been formed with this enzyme (90); this is the first reducing disaccharide reported to be formed by the enzyme. Arsenate has been substituted for phosphate (91) and found to replace phosphate catalytically, but the arsenate compound breaks down spontaneously; this is analogous to the spontaneous break-

down, without energetic coupling, of phosphoarseno glyceric acid (48, 92). A review discussion of the enzymatically synthesized disaccharides is found in the Neberg memorial number of the *Archives of Biochemistry* (93).

Cellulose formation.—Hehre, Carlson & Neill (94) have reported the formation of starch-like material from glucose-1-phosphate by a number of bacteria, including *Corynebacterium diphtheriae* and several hemolytic streptococci; these compounds gave a blue color with iodine.

Cellulose formation by growing and resting cells of the *Acetobacter* has been reported. Kaushal & Walker (95) demonstrated that the polysaccharide formed from a number of substrates was cellulose as indicated by x-ray diffraction studies. Hestrin *et al.* (96) have shown that resting cells of *Acetobacter xylinum* form cellulose under aerobic conditions in the presence of utilizable carbohydrates. Respiration was required for cellulose formation.

Crystalline enzymes.—The first of the bacterial enzymes has been crystallized. Meyer, Fuld & Bernfeld (97) reported the purification and crystallization of bacterial amylase from *Bacillus subtilis*. The enzyme is an α -amylase similar to that from pancreas.

Herbert & Pinsent (98) have crystallized bacterial catalase from *Micrococcus lysodeikticus*. This enzyme is freed from the cells by lysozyme, precipitated by alcohol, and finally crystallized from ammonium sulphate. The bacterial enzyme is similar to the catalase enzyme crystallized from horse, cow, and sheep blood. On the basis of the recoveries, it is estimated that the cells contain about 2 per cent catalase on the dry weight basis; molecular weight estimations indicate the presence of about twenty thousand molecules of catalase per cell.

Hydrolytic enzymes.—Zamecnik & Lipmann (99) have demonstrated that lecithin interferes with the combination of *Clostridium perfringens* antitoxin and the α -toxin, which involves a lecithinase. When the enzyme was first allowed to act on the lecithin the antitoxin failed to inhibit the lecithinase action, though it did gradually decrease the reaction rate. This inhibition is suggested as a possible factor in the lack of success of antitoxin treatment for advanced cases of *Clostridium perfringens* infections.

There have been reports on the production of various hydrolytic enzymes by bacterial cultures. LePage, Morgan & Campbell (100) have reported upon the conditions favoring the production

and purification of penicillinase, as have Housewright & Henry (101), who studied the kinetics and properties of this enzyme and reported that penicillinase is an adaptive enzyme produced only in response to the presence of penicillin (102).

Buck (103) has reported on the characteristics of a heat-resistant phosphatase from *Lactobacillus enzymothermophilus*. His work suggests that one factor involved in thermophilic behavior may be greater thermostability of the enzymes. The mechanism of this stability is, however, unknown.

Studies on hyaluronidase enzymes of the clostridia (104) and the streptococci (105) have been reported. The latter were shown to be adaptive.

Lipolytic enzymes of sulfate-reducing anaerobes and of *Clostridium perfringens* have been studied (106).

Site of action of metabolic inhibitors.—Krampitz & Werkman (107) reported inhibition of nucleic acid or nucleotide metabolism by penicillin. The data suggest that the nucleotide pentose is oxidized as follows:



The pentose level in the cells decreased during respiration; but, in the presence of penicillin, the respiration was inhibited and pentose did not disappear. Ribose and ribose-5-phosphate are not metabolized by intact cells, but ribonucleic acid is decomposed to the extent of 80 to 90 per cent in accordance with the reaction suggested above.

Geiger (108) has reported the relationship of the antibacterial action of quinones and hydroquinones to inactivation of the sulfhydryl groups of the enzymes. Gram negative organisms are usually affected less than gram positive organisms; with the gram negative cultures the addition of sulfhydryl compounds will also reactivate the enzymes.

ASSIMILATION

Amino acids.—A beginning on the mechanism of amino acid assimilation by bacterial cells has been made by Hotchkiss (109), who showed with *Staphylococcus aureus* that, in the presence of an oxidizable substrate, amino acids were assimilated and converted to peptides. In the presence of glucose alone, oxidation occurred and inorganic phosphate was esterified, whereas the addition of

amino acids decreased the amount of inorganic phosphate taken up in relation to the number of acids added. During assimilation, the nitrogen content of the cells was increased as much as 25 per cent and in addition some polypeptides were liberated into the medium.

Gale (110) reported the assimilation of amino acids by resting cells of *Streptococcus faecalis*. Free lysine, glutamic acid, ornithine, and histidine were demonstrated to occur within the cells. An energy source is required for glutamate assimilation, but lysine enters the cell by diffusion; neither amino acid will diffuse out of the cell in the absence of an energy source. The concentrations of these amino acids within the cells as compared with the medium have been studied and relatively great differences in concentration have been found to exist across the cell membrane, differences as great as 500 to 800 μ l. (22 to 36 μ moles) per ml. of the amino acids. Arginine will partially substitute for glucose as an energy source for the assimilation of glutamic acid. Tentative data indicated that aspartic acid behaves in a fashion similar to glutamic acid.

Further, Gale & Taylor (111) found a series of surface active agents, including tyrocidin and several detergents, which allow the loss of amino acids from the internal environment of the cell and have suggested that this leakage may account for the disinfectant action of the agents. Taylor (112) studied the assimilation of amino acids by several organisms and reported a correlation between gram reaction and the accumulation of amino acids by assimilation. The gram positive cocci accumulated glutamic acid, whereas gram negative rods did not.

Gale (113) has studied the influence of penicillin on the assimilation of various amino acids in *Staphylococcus aureus*, including penicillin-resistant strains of this organism. It was suggested that if assimilation were inhibited, the level of glutamic acid within the cell would decrease, whereas, if the metabolism of the cell were inhibited, the level of glutamate within the cell would increase. In the presence of penicillin, the level of the free glutamic acid within the cell decreased, thus indicating that assimilation was inhibited. In this case also it was suggested that failure of the cells to grow resulted from starvation due to a depletion of the amino acid level of the cells. A concentration of penicillin which will inhibit glutamic acid assimilation is comparable to that required to prevent growth.

The utilization of ammonia by *Serratia marcescens* was studied by McLean & Fisher (114), who showed with resting cells that the addition of ammonia increased the respiration rate and that the higher rate was maintained until the ammonia was utilized, approximately two moles of oxygen being required per mole of ammonia assimilated. Analysis of the suspending medium and the cells after the period of assimilation showed that the ammonia was incorporated into cellular material. Thus a gram negative organism capable of synthesizing amino acids used respiration energy to assimilate ammonia in a manner analogous to the assimilation of amino acids by gram positive organisms in the presence of an energy source (109, 110).

Assimilation of carbon compounds.—White & Werkman (115) studied the assimilation of labeled acetate by yeasts in an attempt to determine if the acetate was converted to fat and fatty acids. They found that isotopically labeled acetate added to growing cultures stimulated the storage of the isotope in fat; the fat content of the cells was approximately doubled in the presence of acetate. Only a trace of labeled carbon was found in lactate and this was in the carboxyl group. Isotopically labeled carbon dioxide was not converted to fat; thus, acetate is used directly and not via carbon dioxide. White, Krampitz & Werkman (116) have also studied the assimilation of acetate by yeasts.

Brockmann & Stier (117) have studied the effect of sodium azide on the fermentative ability of various yeasts. The addition of 0.002 *M* azide increased the rate of yeast fermentation and at the same time interfered with assimilation and the storage of energy-rich phosphate. These workers suggest that the influence of azide on fermentation is similar to that of arsenate.

The stimulation of bacteriophage formation by indole-3-acetic acid, as demonstrated by Cohen & Fowler (118, 119), may be another assimilatory process. However confirmation of the effect is still needed.

AMINO ACID SYNTHESIS AND BREAKDOWN

Although the nitrogen fixation problem will be reviewed by Virtanen in Volume II of the *Annual Review of Microbiology*, it may be pertinent to mention here that Burris & Wilson (120) have now reported the utilization of ammonia as an intermediate in nitrogen fixation and thus in amino acid synthesis by *Azotobacter*.

The utilization of ammonia by *Serratia marcescens* during endogenous respiration also appears to involve amino acid synthesis [McLean & Fisher (114)].

Freeland & Gale (121) have analyzed a number of bacteria and yeasts for amino acid content and have found that for those amino acids which may be determined by the decarboxylase method, the medium does not significantly affect the composition of the cells. The amino acid content of gram positive and gram negative organisms is similar with the possible exception of arginine, which comprises about 10 per cent of the total nitrogen of the gram negative rods and about half this level in gram positive cocci. Several bacilli were tested and found to lie midway between these in arginine content.

Synthesis of methionine and cystine.—Lampen, Jones and co-workers (122, 123) have contributed to the mechanism of synthesis of the sulfur-bearing amino acids by *Escherichia coli*. Most of these studies were carried out either with ultraviolet-produced mutants or with metabolic inhibitors. Their work showed that methionine can be replaced by its keto acid analogue or by homocysteine.

For *Escherichia coli*, methionine (123) could be partially replaced by norleucine; possible mechanisms were suggested (5). Other studies (124), employing mutants to determine the route of amino acid synthesis from sulfate, led to the suggestion that the sulfate is reduced to hydrogen sulfide, followed by the reversal of cysteine desulfurase to form cysteine. No data are available, however, on this point. Possible routes of synthesis, involving cysteine, cystathione, homocysteine, and methionine, were also presented. However, these suggestions must remain tentative until further data are available.

Lampen & Jones (125) also discussed the action of *p*-amino-benzoic acid and pteroylglutamic acid in the synthesis of methionine and lysine.

Horowitz (126) has used *Neurospora* mutants to study the synthesis of sulfur-containing amino acids. With these mutants it appeared that the route of synthesis is cysteine, cystathione, homocysteine, and methionine.

Methionine synthesis is inhibited by 2-chloro-*p*-aminobenzoic acid and by sulfanilamide; these inhibitors act at different points in the synthetic mechanism since strains resistant to 2-chloro-*p*-aminobenzoic acid were still sensitive to sulfanilamide, while cells

resistant to sulfanilamide were 2-chloro-*p*-aminobenzoic acid-resistant (127).

Interconversion of amino acids.—With *Torulopsis utilis*, C^{14} in the carboxyl group of alanine was released as respiratory carbon dioxide, whereas carboxyl-labeled glycine was converted to serine and proline as well as to respiratory carbon dioxide (128). Therefore, the formation of glycine from serine is reversible [Shemin (129)].

Tryptophane.—During the past year, two groups of workers have contributed to the studies on tryptophanase and have given some clarification to the mechanism of action of this enzyme. Dawes, Dawson & Happold (130), continuing their studies, have shown that about 25 per cent of the tryptophanase present in acetone-dried preparations may be obtained in a cell-free state. These extracts were inactivated by dialysis and reactivated by recombination of the enzyme and the dialyzing fluid. Further studies by these workers (131) showed the effect of a series of inhibitors on the enzyme action. In agreement with Tatum & Bonner (132), indole production was found to be inhibited by the presence of serine (133). Both serine and alanine were oxidized more rapidly by the enzyme system than was tryptophane. Evidence was obtained for the accumulation of alanine during tryptophanase action in the presence of mepacrine, to inhibit flavine-mediated respiration. The partially resolved enzyme was reactivated in the presence of pyridoxal phosphate, riboflavin, and diphosphopyridine nucleotide.

Wood, Gunsalus & Umbreit (134) isolated a cell-free tryptophanase from *Escherichia coli*. Their preparations catalyzed the breakdown of tryptophane according to the following reaction:



The enzyme was completely resolved and found to be reactivated by pyridoxal phosphate. The system was somewhat sensitive to cyanide, possibly due to the carbonyl group of the coenzyme. As neither serine nor alanine was broken down by the cell-free enzyme preparation, they do not appear to be intermediates in the reaction.

Fildes & Rydon (135) have studied growth inhibition of *Bacterium typhosum* by a series of substituted tryptophane and indole derivatives.

Sources of amino acids for microorganisms.—Simmonds, Tatum

& Fruton have reported the utilization of a series of amino acid peptides by mutants of *Escherichia coli*. The leucineless mutant of *Escherichia coli*, obtained by x-radiation, has been shown to utilize a series of leucine di- and tri-peptides (136). Peptides which contained either free amino or carboxyl groups of leucine were used as was the penta-peptide in which both were bound; leucinamide and N-acetyl leucine were not active.

Utilization of phenylalanine and tyrosine was also studied (137), and, as in the case of leucine, the peptides replaced the respective amino acids for growth. Several of the peptides were less active on a molar basis than the free amino acids. Phenylpyruvic acid replaced phenylalanine, and *p*-hydroxyphenylpyruvic acid substituted for tyrosine, but the acetyl and dehydro derivatives were not utilized, nor were the D-amino acids active. D-Glutamic acid, as well as L-glutamic acid, is however active for the growth of *Lactobacillus arabinosus* [Dunn *et al.* (138)], both isomers having been found by analysis to be present in the cellular material. On the other hand, *Streptococcus faecalis* and *Leuconostoc citrovorum* did use the D-isomer of glutamic acid for growth.

Fruton and co-workers (139) have tested the ability of *Escherichia coli* to utilize various acetyl dehydroamino acids. It has been postulated that a condensation between acid amides and keto acids followed by reduction might occur as a route for peptide synthesis. Acetyl dehydrotyrosine was not used for growth, but in the presence of tyrosine this compound disappeared with the formation of a substance which has been isolated but not as yet completely identified.

Beadle and co-workers (140) have reported the existence of kynurenine as an intermediate in the formation of nicotinic acid from tryptophane by *Neurospora*.

Imbalance.—Studies on amino acid imbalance were presented by Shive *et al.* (141); β -2-thienylalanine inhibited tyrosine synthesis. Phenylalanine was converted to tyrosine, but tyrosine did not serve as a source of phenylalanine. It was suggested that tyrosine may be formed from phenylalanine by direct oxidation of the aromatic ring, and thus phenylpyruvic acid need not serve as an intermediate. High levels of tyrosine inhibited phenylalanine synthesis (142), very probably as a competitive inhibitor. Tryptophane inhibited the utilization of phenylalanine; the growth of

Streptococcus faecalis, for example, was inhibited by two to ten mg./ml. of DL-tryptophane and the inhibition was reversed by 40 to 100 μ g. of phenylalanine. Thus tyrosine prevented synthesis, whereas tryptophane (143) appeared to compete for the enzymes responsible for phenylalanine utilization.

Other cases of imbalance were shown by the studies of Koser & Kasai (144), who found that high levels of nicotinic acid or nicotinamide inhibited growth. The inhibition could be relieved by yeast extract, but not by a vitamin mixture. The agent responsible for the reversal of the inhibition has not as yet been determined.

THE FUNCTION OF ESSENTIAL METABOLITES

Biotin.—At the close of the past year, it was known that biotin-deficient yeast showed a depressed rate of respiration and fermentation which could be relieved by biotin and ammonia (145) and that aspartic acid exerted a biotin-sparing action for *Torula cremoris* (146). It had also been suggested that biotin might mediate in the utilization of carbon dioxide (147).

During the present year, very important advances have been made in understanding the function of biotin. Stokes *et al.* (148, 149) observed a relationship between biotin and aspartic acid. In the presence of excess biotin, aspartic acid could be eliminated from the medium for most lactic organisms commonly used for microbiological assay, but with limited levels of biotin (0.5×10^{-3} μ g. per 10 ml.) aspartic acid was required for growth. Cells grown in the absence of aspartic acid synthesized this amino acid, as indicated by assays with *Leuconostoc mesenteroides*, which were unable to dispense with aspartic acid in the presence of biotin. Thus, biotin functions in the formation of aspartic acid. Following this lead, Stokes *et al.* could not find an altered rate of transamination in biotin deficiency. Subsequently Lardy, Potter & Elvehjem (150) studied the role of biotin in the growth of *Lactobacillus arabinosus* and observed that oxaloacetate would promote growth on a biotin- and aspartic acid-deficient medium and that bicarbonate would stimulate growth in a biotin-rich medium, thus indicating the function of biotin in the carbon dioxide fixation step. Other organic acids, including pyruvate, succinate, fumarate, malate, and α -keto glutarate, did not increase growth. In the same journal Shive & Rogers (151), using the competitive inhibitor approach,

reported a relief of biotin inhibition by α -keto glutarate—an observation which was interpreted as indicating that decreased biotin synthesis affected first α -keto glutarate formation.

Shive & Rogers cited a personal communication from Garrison & Eakin, which indicated that the function of biotin involves the formation of oxaloacetate from carbon dioxide and pyruvate during oxidation by yeast.

A new inhibitor of biotin synthesis (2-oxy-4-imidazolidine-caproic acid) (152) is reported by Rogers & Shive; since prevention of toxicity was competitive with desthiobiotin, but noncompetitive with biotin, it was suggested that the point of inhibition was between the desthiobiotin and biotin.

Lichstein & Umbreit (153) were also able to obtain evidence for the function of biotin in carbon dioxide fixation, using resting cell suspensions of *Escherichia coli*. A technique of aging cell suspensions in acid buffer was devised in which the cells were rendered deficient with regard to their ability to release carbon dioxide in a reaction involving a series of acids including aspartic, fumaric, and malic. A vitamin mixture, or biotin alone, reactivated the enzyme system. The release of carbon dioxide in the presence of a trapping agent for pyruvate led to the observation of the action of biotin in oxaloacetate decarboxylase. Thus the function of biotin in carbon dioxide fixation has been approached from both directions. Lichstein & Umbreit (154) have also suggested the function of biotin in deaminases for aspartic acid, serine, and threonine, based on increased rates of ammonia release. Whether this represents a separate function or merely an extension of the observation of the function in carbon dioxide fixation remains to be determined. Studies with biotin-deficient tissue by Ochoa *et al.* (155) have confirmed the function of biotin in the oxaloacetate decarboxylase of animal tissue, but purification of this enzyme has failed to reveal different biotin levels in the enzyme from deficient and normal tissues.

Axelrod, Hofmann and co-workers (156) have continued studies on the metabolism of oxybiotin by yeast and have concluded that this substance possesses biotin activity per se. A series of biotin analogues has been synthesized and activity tested, both as sources of biotin and as biotin inhibitors (157).

A number of workers have reported the substitution of fatty acids and lipid substances for biotin (158, 159, 160). In addition,

oleic acid has been reported as a growth factor for certain lactic acid bacteria (161, 162). The relationship of the fatty acid substitution to biotin function has not as yet been clarified.

Pantothenic acid.—Novelli & Lipmann (163) have given the title "coenzyme A" to a functional form of pantothenic acid, which serves as the coenzyme for acetylation. A test system for acetylation of aromatic amines by liver preparations has been adapted to the assay of the coenzyme by Lipmann (164). During coenzyme A purification, only traces of B vitamins were found; however, combined treatment with phosphatase and a liver enzyme was shown to liberate pantothenic acid (165).

Based on the observation made as early as 1942 by Dorfman *et al.* (166), namely, that pantothenic acid influenced the rate of pyruvate oxidation by *Proteus morgani*, Novelli & Lipmann (163, 167) incubated deficient cells of *Proteus morgani* with pantothenic acid and observed increased levels of coenzyme A in the cells. A parallel increase in pyruvate oxidation was found. *Lactobacillus arabinosus* was also found to convert pantothenic acid to coenzyme A, over 90 per cent of the pantothenic acid being present in this form. In a survey of natural materials as sources of coenzyme A, Lipmann *et al.* (168) found a general distribution, with the clostridia ranking among the richest sources. It now appears, from the studies of Lipmann *et al.* (169), that coenzyme A functions as the general coenzyme for acetylation, and since acetate is known to occupy an important place in the formation and degradation of fatty acids, and in oxidative pathways for carbohydrates, this coenzyme assumes even greater importance.

Ravel & Shive (170) reported the prevention of pantothenic acid synthesis by cysteic acid, a competitive inhibitor of aspartic acid metabolism. The mechanism suggested is the inhibition of β -alanine formation, which may serve as a precursor of pantothenic acid. β -alanine completely relieves the inhibition by cysteic acid, as does glutamic acid.

Shive *et al.* (171) have also reported a biosynthesis requiring pantothenic acid. The inhibition of *Escherichia coli* growth in the presence of cysteic acid can be reversed by citric, *cis*-aconitic, and α -keto glutaric acids. Oxaloacetic and pyruvic acids are inactive, but the two together give slight activity, as does acetate. From these studies, it is concluded that one of the functions of pantothenic acid is in the condensation of acetate or similar compound

in the tricarboxylic acid system. For *Lactobacillus arabinosus*, oleic acid, in the form of Tween 80, increases the antibacterial index in the presence of cysteic acid, thus suggesting a function of pantothenic acid in acetate condensation to form fatty acids. The interpretation of pantothenic acid deficiency as an interruption in oxidative metabolism at the condensation step is in line with the acetylation studies of Lipmann and co-workers (163, 169).

Vitamin B₆ Group.—In extending the observations of the function of Vitamin B₆ in the formation of tryptophane, Schweigert (172) has studied the use of indole and anthranilic acid for the growth of *Lactobacillus arabinosus*. Pyridoxal and pyridoxamine are essential for the synthesis of tryptophane from either substance; furthermore, serine and acetate were shown to increase the rate of tryptophane synthesis. Thus growth studies with *Lactobacillus arabinosus* has extended the studies with cell-free preparations of *Neurospora*, which indicated the function of pyridoxal phosphate in tryptophane synthesis (173). A further function of pyridoxal phosphate as the coenzyme of the tryptophanase system of *Escherichia coli* has been reported by Wood, Gunsalus & Umbreit (134), as discussed more fully under amino acid metabolism. Dawes, Dawson & Happold (131) have also observed the function of pyridoxal phosphate in the tryptophanase system although their work does not indicate quite so simple a system as that expressed above.

Lyman *et al.* (174) have extended the observations on the substitution of the Vitamin B₆ group for certain amino acids in the growth of lactic acid bacteria and have shown that carbon dioxide is also an important factor.

p-Aminobenzoic acid.—Shive and co-workers (175) by the use of partial sulfanilamide inhibition were able to isolate a purine precursor. Upon relief of the inhibition by p-aminobenzoic acid, the precursor failed to accumulate. The complete synthesis of purines would require ring closure by the addition of one carbon, thus indicating a possible function of PAB in carbon transfer.

Unidentified factors.—Following the studies of Miller *et al.* (176) on the pyruvic acid metabolism of streptococci, O'Kane & Gunsalus (177) have found that *Streptococcus faecalis*, strain 10 Cl, harvested from a synthetic medium adequate for growth, exhibited a depressed rate of pyruvate oxidation unless a factor present in yeast extract was added. Stimulations of tenfold or more were not uncommon. Partial fractionation of yeast extract revealed that the

active factor was adsorbed by charcoal and eluted with alkaline agents. It was soluble in a number of organic solvents and precipitated by certain heavy metals. The extreme stability of this substance to acid, alkali, and heat differentiates it from known factors, none of which has been found to replace it for pyruvate oxidation. Since coenzyme A was first known to function in pyruvate oxidation, it was tested but was not found to be active.

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THE CHEMISTRY OF PENICILLIN

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Organisation of chemical work on penicillin.—After the discovery of the remarkable chemotherapeutic value of penicillin at Oxford (1, 2) intensive chemical investigations were started in a number of laboratories in England and the United States of America. In view of the potential importance of this work for the war effort, publication of the results was restricted by the governments of these two countries, and the publication ban remained in force until the end of 1945. The British chemists formed a committee consisting of representatives of different groups, and dated progress reports, now known as the "PEN" series, were sent to the secretary of the committee in lieu of the normal publications. Most of the British degradation work on penicillin is contained in the PEN reports. A brief review of this phase of the work is in course of publication (3). Towards the end of 1943 the Medical Research Council took over the co-ordination of the chemical work on penicillin and formed the Committee for Penicillin Synthesis which replaced the unofficial committee mentioned above. Dated progress reports termed CPS reports were sent to the Committee for Penicillin Synthesis by the British workers. The American workers communicated their results at intervals to the Committee on Medical Research of the Office of Scientific Research and Development and later sent in regular monthly reports.

These reports were given a letter indicating the laboratory where the work was carried out, followed by a number, e.g., Abbott A 1. Negotiations between the Medical Research Council and the Office of Scientific Research and Development were begun with a view to accelerating the work by regular exchange of information between the American and British chemists, and agreement was reached early in 1944 (4). The first American reports were circulated in England in April 1944. Before this time the British workers had no detailed knowledge of the state of the American work on penicillin. The magnitude of the combined Anglo-American effort on penicillin chemistry can be gauged from the fact that nearly seven hundred reports were sent to the co-ordinating government

organisations. The work in these and the PEN reports deals mainly with (a) degradation studies on penicillin; (b) the synthesis of model compounds for the different structures suggested for the penicillin molecule; (c) the synthesis of degradation products of penicillin and (d) the synthesis of intermediates, for projected syntheses of penicillin.

Copies of the reports have been deposited in some of the principal scientific libraries of Great Britain and in the United States Department of Commerce Office of Technical Services where they are accessible for consultation. A collective account of the work contained in the reports is in process of being published by the Princeton University Press in the form of a comprehensive Anglo-American monograph, under the supervision of the National Academy of Sciences and the Office of Scientific Research and Development (5).

A short statement on the results of the degradation work on penicillin was issued under the auspices of the Committee on Medical Research (O.S.R.D.), Washington, and the Medical Research Council (London) (6).

In this review it is attempted to give a brief summary of the most important results of the work on penicillin chemistry without giving extensive experimental details. Much of the enormous volume of synthetic work achieved has had to be omitted for lack of space and the Anglo-American monograph on the Chemistry of Penicillin (5) may be consulted for fuller details. Most of the literature is quoted by references to the original reports. These will be extensively reproduced in the Anglo-American monograph. It would have been more convenient for the reader to have page references to this publication, which will be more easily accessible than the reports, but it was unfortunately impossible to provide these because of the delay in the publication.¹ For the sake of brevity and clarity no attempt is made to follow strictly the historical course of the development of penicillin chemistry in the presentation of the material. A short historical survey has been published (7) and more extensive accounts are in preparation (8, 9, 10).

Multiplicity of penicillins. Nomenclature.—As the work on the degradation progressed it became clear that penicillin was not a

¹ Republication of the present list of references with cross references to the Anglo-American monograph (5) is contemplated in the next volume of this *Review*.

single substance, but that there existed several penicillins, possessing very similar biological and chemical properties, though differing in their chemical composition. It was found that they contained a common nucleus, but differed in the composition of a side chain R (XXVIII). They were at first distinguished in England by Roman numerals (following the historical sequence of their discovery), and in the United States by letters. To make the nomenclature as far as possible unambiguous it was decided to replace the system of numbers or letters by prefixes indicating the chemical nature of the side chain R. Table I gives a list of penicillins which have hitherto been isolated in the pure state from mould culture filtrates.

TABLE I
LIST OF PENICILLINS

Name of penicillin	Original designation	Formula of side chain	Empirical formula	Reference
Δ^3 -penicillin	I or F	$\text{CH}_2\text{CH}_2\text{CH}=\text{CH} \cdot \text{CH}_2-$	$\text{C}_{16}\text{H}_{17}\text{O}_4\text{N}_2\text{S}$	11, 12
Δ^2 -penicillin		$\text{CH}_2\text{CH}=\text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2-$	$\text{C}_{16}\text{H}_{17}\text{O}_4\text{N}_2\text{S}$	13
n-heptylpenicillin	K	$\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$	$\text{C}_{23}\text{H}_{35}\text{O}_4\text{N}_2\text{S}$	14, 15
benzylpenicillin	II or G	$\text{C}_6\text{H}_5\text{CH}_2-$	$\text{C}_{18}\text{H}_{19}\text{O}_4\text{N}_2\text{S}$	16
p-hydroxybenzylpenicillin	III or X	$\text{OH} \cdot \text{C}_6\text{H}_4\text{CH}_2-$	$\text{C}_{18}\text{H}_{17}\text{O}_5\text{N}_2\text{S}$	17, 18

Amylpenicillin has been prepared by catalytic hydrogenation of Δ^2 -penicillin (19), and it has also been isolated from mould culture filtrates (20). Evidence of the existence of other natural penicillins, as yet not identified, has been obtained by partition chromatography (21) on paper strips and by counter-current distribution between water and different organic solvents (22) in the apparatus of Craig (23). In addition semi-artificial penicillins have been prepared by introduction of different groups into p-hydroxybenzylpenicillin [iodination, diazotation (24)] and also by addition of various derivatives of phenylacetic acid, acting as precursors, to the culture medium (see below).

Purification.—In view of the sensitivity of the penicillin molecule to many reagents the methods for the purification of the penicillins are limited. Among others the following were used: (a) Distribution between water and different organic solvents. This method forms the basis of the purification process used in the commercial production of penicillin. It depends on the fact that the

penicillins, which are monobasic acids, are more soluble in the form of the free acids in organic solvents than in water, whereas the salts are more soluble in water than in organic solvents. It is thus possible to extract the penicillins from the acidified aqueous solution into an organic solvent and to retransfer them into a smaller volume of water (2, 25). Amylacetate, butylacetate and chloroform are the solvents most commonly used for the extraction of the penicillins from aqueous solution, but the partition coefficient of *p*-hydroxybenzylpenicillin between water and chloroform is much smaller than that of the other penicillins, so that it cannot be extracted from aqueous solution by this solvent (18). The penicillins can be extracted from neutral aqueous solution by *n*-butanol; their solubility in water is decreased to a large extent by saturation with ammonium sulphate (26). Data for the partition coefficients for the distribution of benzylpenicillin between water and different solvents have been compiled [(27), see also (22)]. (b) Chromatographic procedures: (i) Chromatography on alumina. Solutions of the free acid in ether or chloroform [Δ^2 -pentenylpenicillin (25)] or of the sodium salt of benzylpenicillin (28) in 95 per cent acetone were percolated through columns of neutralised alumina. (ii) Partition chromatography on silica gel. Modifications of the technique of Martin & Synge (29) were used. Solutions of the penicillins in form of the free acids in organic solvents, usually ether or chloroform, were percolated through columns of silica gel mixed with barium carbonate (30) or phosphate buffer of different pH (12, 31). The method of partition chromatography has proved particularly effective for the purification of the penicillins. (c) Precipitation of penicillin salts with organic bases and ammonia by addition of these agents to solutions of the free acids in organic solvents. It was shown (32) that the triethylamine salt of benzylpenicillin can be obtained in the crystalline state by fractional precipitation of impure preparations (activity as low as 300 units per mg.) with triethylamine from ethereal solution, and this method has been extended to other organic bases [*N*-ethylmorpholine, *N*-ethylpiperidine (33)] and ammonia (34).

Separation of the different penicillins.—The penicillins differ from each other in their partition coefficients between water and ether at pH 5 to 7 and can be separated by means of partition-chromatographic methods (21, 35). When silica-gel phosphate columns are percolated with a mixture of the different penicillins in ethereal solution, *p*-hydroxybenzylpenicillin moves slowest, then

follow benzylpenicillin, Δ^2 -pentenylpenicillin, and *n*-heptylpenicillin in order of increasing rate of development. On the basis of the fact that *n*-heptylpenicillin is considerably more soluble in ether at pH 6 than the other penicillins, and can be washed right through the silica-gel-phosphate column, a method for its quantitative determination has been worked out (35).

Crystalline salts of the penicillins.—Of all metal ions only the alkali salts of the penicillins have been obtained in the crystalline state. The following crystalline metal salts have been prepared. Δ^2 -Pentenylpenicillin: sodium salt (11, 12), rubidium, and calcium salt (36). Benzylpenicillin: sodium salt (28), potassium salt (37), ammonium salt (38). *p*-Hydroxybenzylpenicillin: sodium salt (18). *n*-Heptylpenicillin: sodium salt (39). The following salts of benzylpenicillin with organic bases have been prepared in the crystalline state: triethylamine salt (32), benzylamine salt (40), *N*-ethylpiperidine salt, *N*-methylpiperidine salt, *N*-ethylmorpholine salt (33).

Esters of the penicillins.—The carboxyl groups of the penicillins can be esterified, but because of their great instability the only practicable way to form esters is to react the free acids with diazoalkanes, normally in ethereal solution. Meyer, Hobby & Chaffee (41) were the first to use this method for the preparation of the methyl, ethyl, *n*-butyl and benzhydryl esters of a penicillin mixture of low potency. The methylester of Δ^2 -pentenylpenicillin was prepared from a highly purified barium salt, but was not obtained crystalline (42). The methyl (43, 44) and ethyl (44) esters of benzylpenicillin have been obtained in crystalline state. The benzyl ester (45, 46, 47) and *p*-iodobenzyl ester (48) of benzylpenicillin were prepared, but only in the amorphous state. The esters of the penicillins have only a low antibacterial activity *in vitro*, but *in vivo* they were found to be as active as the sodium salts (41) and the benzyl ester even more active (49) possibly because it was excreted less rapidly. The activity of the esters *in vivo* is due to their hydrolysis by tissue esterases. An esterase hydrolysing the methylester of benzylpenicillin was shown to be present in extracts of guinea pig livers (50).

PHYSICOCHEMICAL PROPERTIES OF THE PENICILLINS

Solubilities.—In the form of the free acid all the penicillins are readily soluble in alcohols, ketones, ethers, and esters. They are less soluble in aromatic hydrocarbons and insoluble in aliphatic

hydrocarbons. Of the chlorinated hydrocarbons chloroform is the best solvent though *p*-hydroxybenzylpenicillin cannot be extracted by it from aqueous solution. The penicillins in form of the free acids are only sparingly soluble in water in which they rapidly lose their biological activity. Of the metallic salts of the penicillins only those of the alkali and alkaline earth metals (of the latter in particular the calcium and barium salts) have been studied in detail. They are very soluble in water, and practically insoluble in ether, chloroform, and amylacetate. The sodium salt of benzylpenicillin in the amorphous state is very soluble in acetone and ethylacetate, but precipitates from these solvents in the crystalline state after a short time interval. In the crystalline state the sodium salt of benzylpenicillin is soluble in methanol, less soluble in ethanol and sparingly soluble in *n*-butanol. It is practically insoluble in acetone and ethylacetate. Small amounts of water increase enormously its solubility in these solvents. The alkali salts of the penicillins are hygroscopic in the impure state, but not when crystalline. The calcium and barium salts of the penicillins are not hygroscopic even when impure.

Optical activity.—The penicillins are strongly dextrorotatory. Thus $[\alpha]_D^{20}$ of the sodium salt of benzylpenicillin was $+290^\circ$ to $+300^\circ$ (51).

Ultraviolet absorption.—The penicillins with aliphatic side chains have no characteristic absorption in the ultraviolet region, showing only a nonspecific end absorption below 2600 Å. Benzylpenicillin shows the ultraviolet absorption characteristic of the phenyl group, with peaks at 2644 Å and 2586 Å [for complete absorption curve made with material used for the international penicillin standard see (52)]. *p*-Hydroxybenzylpenicillin has a peak at 2780 Å (18) and exhibits the characteristic behaviour of the phenolic group in the ultraviolet spectrum, showing strongly increased extinction in the alkaline range, due to the ionisation of the phenolic hydroxyl group (18a).

Infrared absorption.—The infrared spectrum has been determined for benzylpenicillin in form of the free acid (53, 54), the sodium salt (53 to 58) and the methylester (53 to 60). The spectrum is characterised by a band near 3.0μ and three strong bands in the range of 5.5 to 6.4μ , one of which can be attributed to the C=O of the carboxyl group. Of the two remaining bands one (5.62μ) is independent of the type of penicillin, state of the car-

boxyl group and the physical state of the material, and the exact position of the other (5.9 to 6.06μ) depends on these factors. It was found to shift to higher values if amorphous material was used. In addition the penicillins exhibit a strong band in the range of 6.47 to 6.63μ , which is attributed to the presence of a monosubstituted amide linkage.

Molecular weight.—The molecular weight of benzylpenicillin has been determined by the cryoscopic method with the sodium salt in water (51) and the methylester in benzene (61). The values found correspond to the simple formula $C_{16}H_{18}O_4N_2S$ (molecular weight 334).

Polarographic behaviour.—The behaviour of sodium benzylpenicillin in the polarograph has been studied by several groups of investigators (62, 63). With the salt freshly dissolved in water, the polarographic curve was indistinguishable from the blank. When the solution was allowed to stand, a catalytic cysteine-like wave developed gradually. This effect was greatly accelerated by cobalt.

Acid-base properties.—The penicillins are monocarboxylic acids, the carboxylic group possessing a pK value of about 2.8 (64, 57). Evidence for the titratable centre being an acidic and not a weak basic group was obtained (64) from the fact that the pK value shifted to 4.8 in 80 per cent alcohol and was not appreciably altered by changes in temperature. No evidence of the presence of a basic group, even of a very weak type, in the penicillin molecule was obtained (25, 64). Titrations of benzylpenicillin (65) with sulphuric acid in glacial acetic acid showed that one equivalent (corresponding to the carboxyl group) was taken up when the titrations were carried out rapidly; when carried out slowly, absorption of more than one equivalent of sulphuric acid took place, due to the formation of decomposition products.

CHEMICAL PROPERTIES

Inactivation of the penicillins.—The penicillins are characterised by the ease with which they lose their antibacterial activity under the influence of many reagents. They are inactivated in aqueous solution at a pH range below 4 and above 9 at room temperature (2, 25), by primary alcohols and amines (25), by many metal ions such as zinc, copper, cadmium and mercury (25), by carbonyl reagents such as hydrazine and hydroxylamine (25), cysteine (66, 67), thiocyanic acid (68, 69) and acetic acid (70, 71).

The nature of reactions occurring under the influence of these reagents will be discussed below.

Stability.—In form of the free acid the penicillins preserve their antibacterial activity unchanged for several hours at room temperature when kept completely dry (72, 73). The free acids are, however, very hygroscopic and are inactivated rapidly by the presence of a small amount of water. In organic solvents which do not react with the penicillins the free acids are stable at room temperature for indefinite periods, and solutions in amyl acetate can be concentrated in vacuo at 40 to 50°C. without loss of activity. In the dry state the alkali and alkaline earth salts of the penicillins are stable for indefinite time periods. Some commercial preparations were found to lose their antibacterial power, even when kept dry; this was due to impurities. In one case the inactivating substance was identified as *p*-hydroxyphenylacetic acid (74). The crystalline sodium salt of benzylpenicillin did not lose activity when kept at 60°C. for six weeks (75). The crystalline salt of benzylpenicillin is stated to be more resistant to heating at 60° and 100°C. than the amorphous preparation (76). Whereas the amorphous preparation was completely inactivated after heating for one day at 100°C. the crystalline preparation retained 80 per cent of its activity after heating for ten weeks at 100°C. In aqueous solutions the penicillins are stable in the pH range of 5 to 8. Figures for the "half life time" of pure sodium benzylpenicillin at different pH values and temperatures have been given (77). The sodium salts of the penicillins appear to be more stable in buffer than in water (78). A stabilising effect of phosphate buffer (79, 80), hexametaphosphate (81)⁻ and citrate buffer (82) was observed. The sodium salt of benzylpenicillin is stable in liquid ammonia for at least one hour (78). The effect of azlactonising agents on the stability of benzylpenicillin in the form of the free acid, the sodium salt and the methylester has been investigated. Benzylpenicillin was stable in dry ether to acetic anhydride in pyridine for fifteen hours at 25° (70), to benzoylchloride and acetic anhydride in aqueous solution in presence of sodium bicarbonate, under the conditions of the Schotten-Baumann reaction (70), and to ketene in ethereal solution (73); the methylester of benzylpenicillin did not react with carbobenzoxychloride in the presence of pyridine after fifteen minutes at 0 to 5°C., or with benzoylchloride, phenylisocyanate or acetic anhydride in the presence of pyridine at 30° after

one hour (83), or with ketene after thirty minutes at 20°C. (84). On boiling the methylester of benzylpenicillin for one minute in pyridine with benzoylchloride and subsequent treatment with alcoholic potash the methylester of α -*N*-benzoyl, β , β -dimethylacrylic acid was obtained (85).

Presence of active hydrogen.—Determinations of active hydrogen with methylmagnesium iodide according to Zerewitinoff on the methylester of benzylpenicillin showed the presence of one active hydrogen atom (61, 86). Determination of labile hydrogen by exchange with deuterium on the sodium salt of benzylpenicillin gave the same value (87).

Catalytic hydrogenation.— Δ^2 -Pentenylpenicillin readily takes up hydrogen in the presence of platinum oxide (19, 25, 88), the Δ^2 -pentenyl side chain being reduced to amyl. Amylpenicillin is stated to be biologically more active than Δ^2 -pentenylpenicillin (89). In the presence of a large excess of platinum oxide sodium benzylpenicillin in phosphate buffer (pH 7) takes up three molecules of hydrogen. This is attributed to the hydrogenation of the phenyl group to a cyclohexyl group (88). During hydrogenation the larger part of the biological activity is lost.

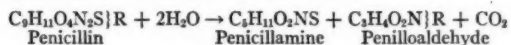
Effect of aluminium amalgam.—The penicillins are stable to this reducing agent in aqueous solution at pH 7, and it has been used in a process for purifying Δ^2 -pentenylpenicillin (25).

Effect of oxidising agents.— Δ^2 -Pentenylpenicillin is rapidly oxidised by oxidising agents attacking double bonds, such as hydrogen peroxide and permanganate (25). Benzylpenicillin, however, is remarkably stable towards oxidising agents. In the form of the free acid in acetic anhydride solution it is not attacked by chromic oxide at room temperature for forty-five minutes, while the sodium salt is not inactivated by potassium dichromate in aqueous solution at pH 7 (90). Free benzylpenicillin is not inactivated by hydrogen peroxide after treatment for fifteen minutes in tertiary butanol at 75°C. (90), and the methylester was recovered unchanged after treatment with hydrogen peroxide for three days at room temperature in 60 per cent acetone (71), but the sodium salt was rapidly oxidised in aqueous solution with the development of carbon dioxide and penilloaldehyde. The methylester of benzylpenicillin is rapidly attacked by selenium dioxide (91), as indicated by a rapid fall in optical rotation. The sodium salt of benzylpenicillin is slowly oxidised by potassium

permanganate at pH 7 in aqueous solution, while the methyl-ester when treated at 25°C. in a mixture of dioxane and phosphate buffer at pH 6.8 with potassium permanganate is converted into the sulphone [(92) see below]. With sodium periodate the methyl-ester of benzylpenicillin is converted into the sulphoxide (93). The penicillins are stable towards iodine in aqueous solution at neutral pH for several hours. When the sodium salt of benzylpenicillin was allowed to stand in the presence of iodine for prolonged time periods, iodine was gradually taken up (94). Bromine water is rapidly decolourised by the penicillins in aqueous solution because of the oxidation of breakdown products formed under the influence of acid. The methylester of benzylpenicillin takes up two atoms of bromine in chloroform (95).

THE FUNDAMENTAL COMPONENTS OF THE PENICILLIN MOLECULE

The penicillin molecule is built up of three basic components into which it can be hydrolysed with the participation of two molecules of water according to the equation:



PENICILLAMINE

Penicillamine, $\text{C}_6\text{H}_{11}\text{O}_2\text{NS}$, is the component common to all penicillins.

Isolation.—Penicillamine is obtained in the form of the hydrochloride from natural penicillin: (a) by hydrolysis with dilute acid at 100° for one hour, followed by precipitation with mercuric chloride, and decomposition of the mercuric complex (96); (b) by inactivation of the penicillins at alkaline pH followed by precipitation with mercuric chloride (97).

Structure.—Penicillamine has the properties of a thiol α -amino acid (31). It gives the colour reactions for free thiol and α -amino groups and all its nitrogen reacts as α -amino nitrogen in the Van Slyke determination. Electrometric titration shows the presence of three ionisable centres, with pK values of 1.8, 7.9, and 10.5 corresponding to the carboxyl, α -amino and thiol groups. The α -amino and thiol groups are in juxtaposition; this is indicated by the facile formation of thiazolidines with ketones and aldehydes. The thiazolidines are decomposed by mercuric chloride. C-methyl determination according to Kuhn-Roth gave a value of

about 0.2 molecules, indicating the absence of a terminal methyl group. On oxidation with bromine the corresponding sulphonic acid $C_5H_{11}O_6NS$ was obtained. On the basis of the above evidence the structure of β -thiolvaline $(CH_3)_2C-CH\cdot COOH$ was proposed



for penicillamine (98) which was proved correct by synthesis (99).

Stereochemistry.—Natural penicillamine belongs to the D-series. This was shown by conversion of the phenylureido derivative to the phenylureido derivative of D-valine by treatment with Raney nickel (72). D-Penicillamine hydrochloride has a very small positive rotation in water (97, 100). L-Penicillamine was shown to be slightly laevorotatory in the presence of hydrochloric acid and to become strongly dextrorotatory ($[\alpha]_D + 63^\circ$) in the presence of sodium hydroxide. This behaviour is typical for amino-acids of the L-series (101). In the presence of 5 per cent potassium phosphate the rotation remained constant within a pH range of 2.8 to 6.9 (102). The isopropylidene derivative of D-penicillamine is dextrorotatory (97, 100).

Derivatives.—Penicillamine can be oxidised to the disulphide by shaking in air in the presence of ferric chloride (103) or by iodine (31). The disulphide is more soluble in water than cystine and more resistant to reducing agents; e.g., it does not give a nitroprusside reaction when treated with cyanide (103). It can be reduced to penicillamine by sodium and liquid ammonia, but not by sodium sulphite (103). It is not attacked by amino acid oxidases in liver and kidney (31).

The methylester of D-penicillamine has been obtained from the methylester of Δ^2 -pentenylpenicillin by treatment with mercuric chloride in aqueous methanol (42) and from the methylester of benzylpenicillin by the same treatment (104). The esters can be prepared from penicillamine by the conventional methods of esterification of amino acids. D-Penicillamine methylester has been prepared in good yield by the action of dimethylsulphite on D-penicillamine hydrochloride (105).

Various N-acyl derivatives of D,L-penicillamine and the optical isomers have been prepared by acylation of S-benzylpenicillamines and subsequent removal of the benzyl groups by sodium and liquid ammonia, and by treatment of penicillamine with the calculated

amount of acid azides in aqueous solution. *N*-Formyl-D-penicillamine methylester has been prepared by treatment of D-penicillamine methylester with formic acid and acetic anhydride (106).

Penicillamine reacts readily with many aldehydes and ketones to give the corresponding thiazolidines. Numerous compounds of this type have been prepared in different laboratories by warming the components with or without solvents, or by warming the acetals and penicillamine hydrochloride. Compounds containing hydroxymethylene groups do not give thiazolidines, but the amino and sulfhydryl groups of penicillamine react independently, the amino group reacting preferentially.

N-acyl penicillamines are readily converted into thiazolines on saturation of ethereal solutions with dry hydrochloric acid (105, 107).

Neither the L- nor D- forms of penicillamine are attacked by oxidative enzymes in liver or kidney extracts (31).

Syntheses of D,L-penicillamine.—D,L-Penicillamine has been synthesised by the following methods: (a) Addition of benzylmercaptan to 2-phenyl-4-isopropylidene-oxazolone (99); (b) Addition of hydrogen sulphide to 2-methyl-4-isopropylidene-oxazolone (108); (c) Addition of thiolacetic acid to α -*N*-benzamido- β , β -dimethylacrylic acid (72); (d) Addition of hydrogen sulphide to 2-ethylmercapto-4-isopropylidene-thiazolone (108a); (e) From α -bromoisobutyraldehyde and benzyl mercaptan by the Strecker synthesis (108a).

Resolution of D,L-penicillamine.—This was achieved by fractional crystallisation of the brucine salts of D,L-formyl-S-benzylpenicillamine (99, 101) and D,L-*N*-formyl-isopropylidene penicillamine (100).

PENILLOALDEHYDES

Occurrence.—While penicillamine is a constituent common to all penicillins each penicillin gives rise to a different penilloaldehyde.

Isolation.—The penilloaldehydes are obtained from acid hydrolysates of the penicillins after treatment with mercuric chloride. Δ^2 -Pentenyl- (109), amyl- (110), heptyl- (39), benzyl- (75), and *p*-hydroxybenzyl- (17) penilloaldehydes have been obtained in this way in form of the 2,4-dinitrophenylhydrazones. Δ^2 -Pentenylpenilloaldehyde has also been obtained in better yield by decom-

position of the alkali inactivation product of Δ^2 -pentenylpenicillin with mercuric chloride in form of its dimedone derivative (97).

Structure: Δ^2 -pentenyl- and amyl-penilloaldehydes.—On the basis of the molecular weight, calculated from crystallographic x-ray data (111) and the analytical figures for the dimedone derivative, the empirical formula for Δ^2 -pentenylpenilloaldehyde was $C_8H_{13}O_2N$. Oxidation with silver oxide gave an acid $C_8H_{13}O_3N$ (112). Information that the American penicillin $C_{16}H_{18}O_4N_2S$ had yielded penicillamine and phenylacetic acid on hydrolysis led the Oxford workers to the assumption that the penilloaldehyde from the American penicillin corresponding to the British penilloaldehyde should have the formula $C_{10}H_{11}O_2N$ and the structure of phenylacetylaminooacetaldehyde, $C_6H_5CH_2CONHCH_2CHO$. It was concluded that the penilloaldehyde $C_8H_{13}O_2N$ was a hexenoyl-aminoacetaldehyde and the acid $C_8H_{13}O_3N$ was hexenoylglycine. Hydrolysis of the acid $C_8H_{13}O_3N$ with acid or alkali liberated α -amino nitrogen and glycine which was isolated and identified crystallographically in the form of its naphthalene- β -sulphonyl derivative (113). Further evidence in favour of the structure of an acylated aminoacetaldehyde was obtained from the fact that heating of the 2,4-dinitrophenylhydrazone of the penilloaldehyde from catalytically reduced Δ^2 -pentenylpenicillin in presence of excess of 2,4-dinitrophenylhydrazine led to the formation of glyoxal osazone (110). Hydrolysis of the penilloaldehyde from catalytically reduced penicillin gave rise to a saturated fatty acid which was identified as caproic acid, isolated in form of the *p*-bromophenacyl ester. The structure of this penilloaldehyde was thus proved as *n*-caproylaminoacetaldehyde (114). The position of the double bond in the hexenoic acid of the penilloaldehyde from Δ^2 -pentenylpenicillin was located in the β,γ -position by oxidation with permanganate which yielded propional. Thus the structure of Δ^2 -pentenylpenilloaldehyde was proved to be β,γ -hexenoyl-aminoacetaldehyde (113).

Structure: benzylpenilloaldehyde.—The structure of this aldehyde was deduced as phenylacetylaminooacetaldehyde from the fact that it had the composition $C_{10}H_{11}O_2N$ and yielded phenylacetic acid on hydrolysis (75).

*Structure: *n*-heptyl penilloaldehyde.*—The structure of this penilloaldehyde, composition $C_{10}H_{19}O_2N$, as *n*-capryloylaminoacetaldehyde was proved by comparison with synthetic material (15).

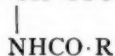
Structure: *p*-hydroxybenzylpenilloaldehyde.—This penilloaldehyde had the composition $C_{10}H_{11}O_3N$ and its structure as *p*-hydroxyphenylacetylaminooacetaldehyde was proved by the isolation of *p*-hydroxyphenylacetic acid (17).

Reactions.—The penilloaldehydes are fairly stable in neutral aqueous solution, but are rapidly destroyed in alkaline solution. They are hydrolysed into their components by mineral acids at 100° . The acetals of the penilloaldehydes are much more stable than the free aldehydes; in contrast to other acetals they show a remarkable resistance to treatment with acids. On warming the acetals with the hydrochlorides of penicillamine and cysteine or the esters of these amino acids the corresponding thiazolidines are formed with great ease.

Syntheses.—The most frequently used method for the preparation of the acetals of the penilloaldehydes has consisted in the acylation of aminoacetal with the appropriate acid chlorides.

THE LABILE CARBOXYL GROUP

All penicillins contain a labile carboxyl group which is liberated in the form of carbon dioxide (25) when they are hydrolysed at 80° to 100° for ten to thirty minutes or when the products of inactivation with acid or alkali are treated with mercuric chloride at room temperature (31, 109). This labile carboxyl group is present in bound form in the penicillin molecule and is not the carboxyl group of penicillamine. This was shown by decomposition of the methylester of Δ^2 -pentenylpenicillin with mercuric chloride which led to evolution of carbon dioxide and the formation of penicillamine methylester (42). It was clear that the labile carboxyl group must be attached to the penilloaldehyde moiety of the penicillin molecule and the ease of its liberation suggested that it was in the β -position to the aldehyde group, i.e., formed part of a β -aldehydoacid of the structure $CHO \cdot CH \cdot COOH$. This acid was



termed penaldic acid (115).

PENALDIC ACIDS

The free penaldic acids are not stable, being rapidly decarboxylated to the penilloaldehydes. However, derivatives of penaldic acid such as the methylester and the benzylamide have been isolated from the penicillins.

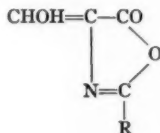
Isolation.—Treatment of benzylpenicillin with benzylamine led to a crystalline product which was biologically inactive and had the composition $C_{30}H_{38}N_4O_4S \cdot H_2O$ (116). Degradation of this product with mercuric chloride gave the benzylamide of penaldic acid (115) which was isolated in form of its 2,4-dinitrophenylhydrazone. Similarly, the product obtained on inactivation of benzylpenicillin with methanol gave, on treatment with mercuric chloride, the methylester of penaldic acid (115), also isolated in form of the 2,4-dinitrophenylhydrazone.

Structure.—Conclusive proof for the structure of methylbenzylpenaldate and the benzylamide of benzylpenaldic acid was obtained by catalytic reduction with Adam's catalyst of the former



to cyclohexylacetylalanine, $C_6H_{11}CH_2 \cdot CONH \cdot CH \cdot COOH$, and of the latter to the cyclohexylamide of cyclohexylacetylserine, $C_6H_{11}CH_2 \cdot CONH \cdot CH(CH_2OH)(CONHCH_2 \cdot C_6H_5)$ (115).

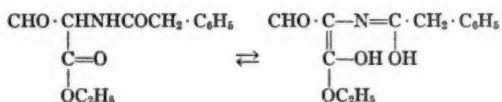
Syntheses.—Of a number of syntheses for the D,L-penaldic acids and their derivatives the following may be mentioned: (a) formylation of *N*-acylated glycine esters with ethylformate and sodium ethoxide, (b) acylation of β,β -diethoxyalanine, $(C_2H_5O)_2CH \cdot CH(NH_2) \cdot COOH$, prepared by C-formylation of *N*-formylglycine-ester and removal of the *N*-formyl group by methanolic hydrochloric acid, or by amination of α -chloro, β,β -diethoxypropionic acid. (c) alcoholysis of oxazolones of the type,



prepared by treatment of *N*-acylated glycine esters with orthoformic ester and acetic anhydride. For experimental details of the best methods of preparation the chapter on penaldic acids and penilloaldehydes in the Monograph on Penicillin Chemistry may be consulted (117). Numerous penaldic acids with different acid radicals have been prepared.

Resolution.—The dimethylacetal of benzylpenaldic acid was resolved by fractional crystallisation of its brucine salt (118). The dextrorotatory form was obtained in almost pure state.

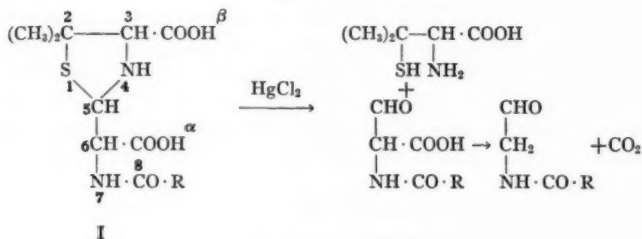
Reactions.—The aldehyde group of the penaldic acids is capable of reaction in the aldehyde and the hydroxymethylene form. The penaldic acid esters and amides react readily with penicillamine and cysteine to give the corresponding thiazolidines (penicilloic and desdimethylpenicilloic acid derivatives—see below). With primary amines such as benzylamine and aniline they give Schiff bases. The benzylamine derivative, a well-crystalline compound (62, 86), reacts in acid solution like the free aldehyde and has been used extensively in synthetic studies. The sodio derivatives of the penaldates react with acid chlorides to give acyl derivatives; several aroyl derivatives (119) and the acetyl derivative have been prepared (120). Ethyl penaldate shows no strong ultraviolet absorption in methanol, but on addition of sodium hydroxide a strong band (E_M 15,000) at 2675 \AA appears which disappears again on acidification. This suggests the existence of a tautomeric equilibrium (121):



A great deal of work on the azlactonisation of different penaldic acids was done in connection with attempts to synthesise the thiazolidine-oxazolone structure of penicillin. Methods for the preparation of 2-benzyl-4-hydroxymethylene oxazolone by cyclisation of benzylpenaldic acid diethylacetal and dimethylacetal have been worked out (122, 123, 124).

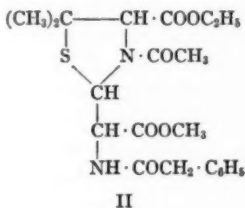
PENICILLOIC ACIDS

After inactivation of the penicillins by alkali a new strong acid group and a basic group, pK about 5, appears (25). The product of inactivation does not give a reaction for free thiol and amino groups, nor can the presence of free penilloaldehyde be detected. On addition of mercuric chloride the mercuric complex of penicillamine is precipitated, and simultaneously penilloaldehyde and carbon dioxide are liberated. This reaction indicated that in alkali inactivated penicillin penicillamine and penaldic acid were linked in a thiazolidine structure (I) which was broken down into its components by mercuric chloride.



The thiazolidines possessing structure I were termed penicilloic acids (115).

Nomenclature.—The penicilloic acids deriving from different penicillins are distinguished by prefixes indicating the nature of the side chains, the compound where R equals hydrogen being termed simply penicilloic acid. The carbon atoms in the penicilloic acids are numbered as indicated in formula I. The two carboxyl groups are termed α and β . The penicilloic acids can be derived from D, DL, or L-penicillamine; the configuration of the penicillamine from which they are derived is indicated by adding D, DL or L to their name. If derived from D-penicillamine, the penicilloic acids can exist in 4 stereoisomeric forms which are designated as α , β , γ and δ (84). The γ -isomer of structure II, for example, would be designated as α -methyl, β -ethyl N^1 -acetyl-D- γ -benzylpenicilloate.



Formation.—Salts of D-penicilloic acids are formed from the salts of the penicillins by the action of alkali (pH 10 to 12), the action of bisulphite (125), the action of copper ions (126), and the enzyme penicillinase (127). α -Esters and α -amides are formed under the influence of primary alcohols and amines (115). The action of hydroxylamine leads to the formation of α -hydroxamic

acids (128, 129), of hydrazine to the formation of α -hydrazides (130), and of cysteine to the formation of α -peptides with the SH group free (126). The alcoholysis of the penicillins is catalysed by copper, tin, and zinc ions and can be greatly retarded by dimer-capto propanol (BAL) (131). Action of acetic acid on benzylpenicillin leads to the formation of *N*⁴-acetyl-D-benzylpenicilloic acid (126, 132).

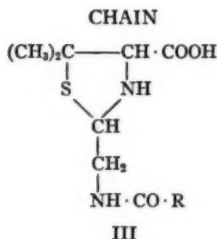
Isolation.—Among others the following derivatives of D-benzylpenicilloic acid have been obtained in the crystalline state by degradation of benzylpenicillin: the monosodium salt of D- α -benzylpenicilloic acid (85), by inactivation of sodium benzylpenicillin with sodium hydroxide; the copper salt of D- δ -benzylpenicilloic acid, by the action of copper ions on benzylpenicillin (126); the benzylamine salt of the α -methylester of D- α -benzylpenicilloic acid, by inactivation of sodium benzylpenicillin with methanol (70, 133); the α -methylester of D- α -benzylpenicilloic acid (134); the benzylamine salt of α -benzyl-D- α -benzylpenicilloate, by inactivation of sodium benzylpenicillin with benzylalcohol (84); the α , β -dimethylester of D- α -benzylpenicilloic acid, by treatment with diazomethane of the methanol inactivation product of sodium benzylpenicillin (83); the benzylamine salt of the α -amide and α -methanamide of D-benzylpenicilloic acid, by treatment of sodium benzylpenicillin with aqueous ammonium hydroxide or methylamine (40); and the benzylamine salt of the α -benzylamide of D- α -benzylpenicilloic acid (116).

Structure.—The structure of benzylpenicilloic acid was proved by decomposition with mercuric chloride of the α -methylester and α -benzylamide which led to the isolation of D-penicillamine and derivatives of penaldic acid (115). The structure of the penaldic acids was proved by catalytic hydrogenation (see above). The structure of penicilloic acid was confirmed by synthesis.

Stereochemistry.—The stereoisomerism of the penicilloic acids has been studied extensively, mainly by the Merck group. The problem was given particular attention because it was considered that the use of the correct isomer might be of crucial importance for a synthesis of penicillin based on ring closure of the penicilloic acids. The absolute configuration of the asymmetric carbon atom 3, belonging to penicillamine, is known as D, because the configuration of this carbon atom does not change on degradation, but the

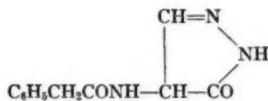
absolute configuration of the other two asymmetric centres (carbon atoms 5 and 6) is not certain. Changes in these centres may occur during the degradation of the penicillins to penicilloates or during the synthesis of penicilloates from penicillamine. Representatives of the four theoretically possible isomers of the penicilloates are known. Natural penicilloates, obtained from benzylpenicillin by the action of alkali and primary alcohols, have the α -configuration. The action of copper on benzylpenicillin leads to the δ -form, but as the δ -isomer can also be obtained by the action of copper on the α and γ -D-benzylpenicilloates (126) it is possible that the α -isomer is the primary reaction product which then undergoes inversion to the δ -form. Synthetic D-benzylpenicilloates, e.g., the α -methyl, α -ethyl, or α , β -dimethylesters, are a mixture of isomers usually containing predominantly the γ -form which crystallises out. α -Methyl-D- γ -benzylpenicilloate undergoes mutarotation on refluxing in methanol for sixteen hours. After removal of the γ -isomer from the mixture of stereoisomers, conversion of the remaining penicilloates first into the benzylamine, then the *l*-ephedrine salts and benzylation in pyridine of the free acids liberated from the salts, the *N*⁴-benzoyl derivative of the α -isomer crystallised from the ethereal solution, while from the mother liquor a small amount of the *N*⁴-benzoyl derivative of the β -isomer was obtained (136). The γ -isomer of α -ethyl-D-benzylpenicilloate was separated from amorphous synthetic material by its insolubility in ether. The α -isomer crystallised from the ether soluble fraction and on benzylation a small amount of the β -isomer was obtained (136). Unlike the α -methylester, the dimethylester of D- γ -benzylpenicilloic acid is stable in pyridine at 100° and pyridine-methanol at reflux temperature (137), but mutarotation of α , β , and γ -forms occurs in methanol-hydrochloric acid, dibutyl-ether-dioxane-hydrochloric acid, acetic acid-perchloric acid and boiling toluene or xylene. Dimethyl-D- α -benzylpenicilloate was obtained by isomerisation of the β - and γ - (138) forms, and the α - and γ - forms of dimethyl-D-benzylpenicilloate could be converted into the β -form (106).

Reactions.—The free penicilloic acids are slowly decarboxylated in aqueous solution at room temperature and more rapidly at higher temperatures (51); the decarboxylated products, thiazolidines of the type III, have been termed penilloic acids.



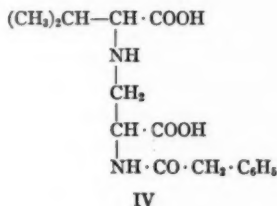
Benzylpenilloic acid was obtained from benzylpenicilloic acid by heating in dry state in vacuo at 78° (40). It can also be prepared directly from benzylpenicillin by refluxing with dilute acids (85, 139); benzylpenicilloic acid may be formed as an intermediate product during the hydrolysis.

The penicilloic acids are zwitterions, possessing two acid groups and one basic group. The pK of the basic group is about 5 in benzylpenicilloic acid (140), while in the α -esters and the α -benzylamide it is about 3.8 (141, 142). The α -esters are readily saponified by one equivalent of alkali at room temperature (78, 143). On treatment of the diesters with one equivalent of alkali the α -ester group is selectively saponified (144, 145); this property has been made use of for the preparation of β -esters. Numerous N^4 -acyl derivatives of benzylpenicilloic acid, its α and β esters, α , β esters and α -amides, have been made (among others the N^4 -formyl, acetyl, isobutyryl, benzoyl, carbobenzoxy, phenoxy and phenylacetyl derivatives). These possess no basic group (121, 128, 146) and resist decarboxylation on heating (147). They are also more stable towards mercuric chloride than penicilloic acid derivatives containing a free imino group; these are readily cleaved into penicillamine and penaldates. The enhanced stability of the thiazolidine ring in N^4 -acylated penicilloic acid derivatives is also indicated by the fact that they do not show mutarotation in boiling ethanol (148). On refluxing α -methyl-D-benzylpenicilloate with hydrazine hydrate in methanol 4-phenylacetyl-amido-5-pyrazolone, is formed (138).



Treatment of α -methyl-D- γ -benzylpenicilloate with sodium nitrite

and hydrochloric acid led to formation of the *N*⁴-nitroso derivative (138, 149). On treatment of dimethyl-*N*⁴-benzoyl-*D*- γ -benzylpenicilloate with methanolic potassium hydroxide α -benzoylamino- β,β -dimethylacrylic acid is obtained (150). Action of potassium permanganate in 50 per cent acetic acid on β -methyl-*N*⁴-isobutyryl-*D*- γ -benzylpenicilloate led to the formation of the sulphone (151). Action of Raney nickel on benzylpenicilloates led to the elimination of sulphur and the formation of desthiobenzylpenicilloates (IV).



During the conversion of penicilloates into desthiopenicilloates one of the three asymmetric centres disappears. Consequently the four stereoisomeric forms of benzylpenicilloic acid give rise to two stereoisomeric forms of desthiobenzylpenicilloic acid. Both forms have been obtained. Treatment of the natural α -benzylamide of *D*- α -benzylpenicilloic acid with Raney nickel gave the α -benzylamide of *D*- α -desthiobenzylpenicilloic acid (85). The α -stereoisomer of desthiobenzylpenicilloic acid was also obtained from desthiobenzylpenicillin (see below). Treatment with Raney nickel of synthetic α -methyl-*D*- γ -benzylpenicilloate gave *D*- γ -desthiobenzylpenicilloic acid (152) and synthetic dimethyl-*D*- γ -benzyl penicilloate gave dimethyl-*D*- γ -desthiobenzylpenicilloate (106). Treatment with Raney nickel of β -methyl-*N*⁴-isobutyryl-*D*- γ -penicilloate gave β -methyl-*N*⁴-isobutyryl-*D*- γ -desthiobenzylpenicilloate (153). The α - and γ -forms of desthiobenzylpenicilloic acid differ in their optical rotation, the α -form being laevorotatory, the γ -form dextrorotatory. Hydrogenolysis of α -ethyl-*D*- γ -benzylpenicilloate and *D*- α -benzylpenicilloate with hydrogen (3000 pounds pressure) and Raney nickel led to the formation of *N*-cyclohexylacetylalanine; hydrogenolysis with Raney nickel at normal pressure gave *N*-phenylacetylalanine (85).

Synthesis.—The synthesis of penicilloic acids is readily accom-

plished by condensation of penicillamine or its esters with penaldates. Numerous penicilloic acids have been synthesised in this manner.

PENILIC ACIDS

The penicillin molecule has the property of readily undergoing intramolecular rearrangements in several ways, leading to the formation of biologically inactive isomers. Early in the chemical studies on penicillin it was noticed that Δ^2 -pentenylpenicillin, on inactivation with acid, was converted into a zwitterion, with the liberation of a new strongly acidic group and a basic group of pK 7.8 (25). The reaction product was isolated in the crystalline state (109, 154), and analysis showed it to be isomeric with Δ^2 -pentenylpenicillin. It was termed penillic acid. Later penillic acids from other acid-inactivated penicillins were isolated.

Isolation.—The free penillic acids have a high power of crystallisation and can be obtained in the crystalline state even from crude penicillin preparations. A convenient method of isolation is to inactivate the penicillins at pH 2 and room temperature, extract the acid solutions with ether to remove impurities, and concentrate *in vacuo*, when the penillic acids crystallise out (109). Yields up to 75 per cent of the theoretical can be obtained under these conditions (11). In addition to Δ^2 -pentenylpenillic acid benzylpenillic acid (72), amylpenillic acid (89) and *p*-hydroxybenzylpenillic acid (17) have been isolated in the crystalline state.

Physical properties.—The penillic acids are strongly dextrorotatory. $[\alpha]_D^{20}$ of Δ^2 -pentenylpenillic acid is $+507^\circ$ to $+527^\circ$, and of benzylpenillic acid is $+500^\circ$ to 548° ; under certain conditions their formation from the penicillins manifests itself by an increase of optical rotation (154). They have a characteristic absorption in the ultraviolet, showing an absorption maximum at about 2380 Å in aqueous solution [for Δ^2 -pentenylpenillic acid see (155), amylpenillic acid (89), benzylpenillic acid (51), *p*-hydroxybenzylpenillic acid (17)]. In form of the free acids the penillic acids are sparingly soluble in all solvents, including water, the *p*-hydroxybenzylpenillic acid being the least soluble.

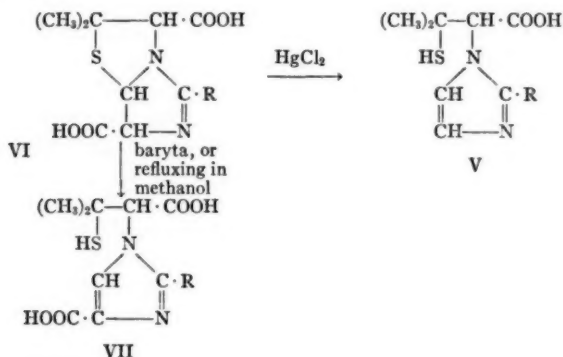
Formation.—The yield of penillic acid from sodium penicillin depends on the acidity of the solution; it is optimal when one equivalent of acid per molecule of sodium penicillin is used. With increasing concentration of acid the yield decreases; in the presence

of 2*N* hydrochloric acid no appearance of the characteristic ultraviolet absorption band of benzylpenillic acid was observed (128). Probably penicilloic acids are formed under these conditions as benzylpenilloic acid, the decarboxylation product of benzylpenicilloic acid, has been isolated from the mother liquors of benzylpenillic acid (156). The methylester of benzylpenicillin, when treated with one equivalent of hydrochloric acid in absolute methanol undergoes the rearrangement to benzylpenillic acid, which was isolated in form of the dimethylester after esterification with diazomethane (157). In the presence of 66 per cent alcohol no formation of benzylpenillic acid took place (146).

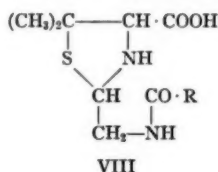
Formation of penillamines and isopenillic acids; structures.—

The penillic acids give no colour reactions for a free thiol group, and no free amino group is present. On refluxing benzylpenillic acid in water for one hour decarboxylation occurred and benzylpenilloic acid was formed (40). On addition of mercuric chloride to the penillic acids in aqueous solution decarboxylation occurs and the mercuric complex of the decarboxylated product is precipitated (109). On removal of the mercury by hydrogen sulphide the hydrochlorides of bases, termed penillamines, are obtained (109). These bases give a strong nitroprosside and ferric chloride colour reaction indicating the presence of a free thiol group. Electrometric titration shows the presence of three ionisable centres: an acidic group, *pK* 2.1; a basic group, *pK* 7.1, which is not an α -amino group, and the thiol group with a *pK* of 10.4 (31). Heating with 2,4-dinitrophenylhydrazine gives the osazone of glyoxal. As the sequence of the atoms in the penilloaldehyde moiety is fixed, only one structure reconcilable with the above facts is possible for the penillamines, the imidazole derivative V (159). The imidazoline structure for the penillic acids follows logically as VI (112); it contains the labile carboxyl group in β -position to a potential aldehyde group which is formed by rupture of the thiazolidine ring under the influence of mercuric chloride. The presence of the imidazole nucleus in the penillic acid molecule was later proved directly by the isolation of 2-benzyl-4-carbomethoxyimidazole after pyrolysis of the dimethylester of benzylpenillic acid at 115° (156, 160). Both benzylpenillic acid and benzylpenillamine lost sulphur easily on treatment with sodium plumbite (161). Benzylpenillic acid, like all *N*-nonacylated thiazolidines, was readily oxidised by iodine in neutral aqueous solution, taking up 5.8 to 6.3 equivalents of iodine

(94). By the action of saturated barium hydroxide on Δ^2 -pentenylpenillic acid an isomeric product, termed iso- Δ^2 -pentenylpenillic acid, was formed (162). This substance was shown to contain four ionisable centres: two acidic groups ($pK_1 < 3$ and $pK_2 3.2$), a basic group ($pK 6.4$), and a thiol group ($pK 10.2$). The presence of a free thiol group was also indicated by a strong nitroprusside reaction. The compound had a weak negative rotation. The properties of the substance were in agreement with structure VII. Benzylpenillic acid was converted into the corresponding iso-benzylpenillic acid by heating in methanol for eighteen to twenty hours (163), by treatment with barium hydroxide at 37°C . for three days (132) and with glacial acetic acid at 23° for twenty-four hours (164), and by refluxing dimethylpenillate in xylene (163). The structures for penillic acid, penillamine and isopenillic acid were confirmed by synthesis.

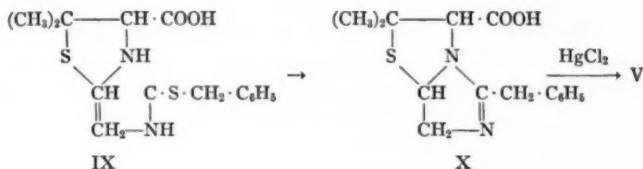


Synthesis: penillamines.—(a) By ring closure of 2-acylamino-methyl-4-carboxy-5,5-dimethylthiazolidines (VIII) with phosphorus oxychloride [$\text{R} = \text{C}_6\text{H}_{11}$ (165); $\text{C}_6\text{H}_5\text{CH}_2$ (166)].

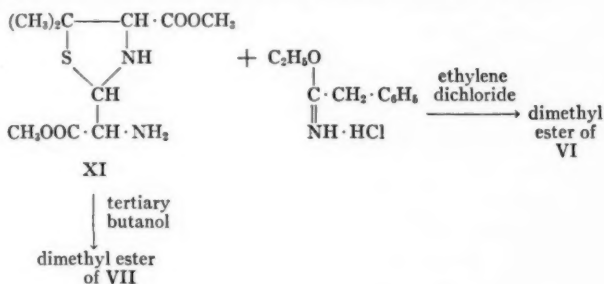


(b) By elimination of hydrogen sulphide from 2-thiophenylacetyl-

aminomethyl-4-carboxy-5,5-dimethyl thiazolidine (IX), obtained by condensation of dithiophenylacetic acid with 2-aminomethyl-4-carboxy-5,5-dimethylthiazolidine, and decomposition of the resulting D,L-decarboxy penillic acid (X) with mercuric chloride (167).



Synthesis: penillic acid.—The dimethylester of benzylpenillic acid was synthesised by condensation of the thiazolidine XI with ethylphenylacetiminoether in ethylene dichloride (106).



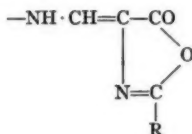
Synthesis: Isopenillic acid.—When the condensation of XI with ethylphenylacetiminoether was carried out in tertiary-butanol, the dimethylester of isobenzylpenillic acid was obtained as the main reaction product (106). The synthetic benzylpenillic and isopenillic acids have the same steric configuration as the natural compounds.

PENICILLINIC ACIDS

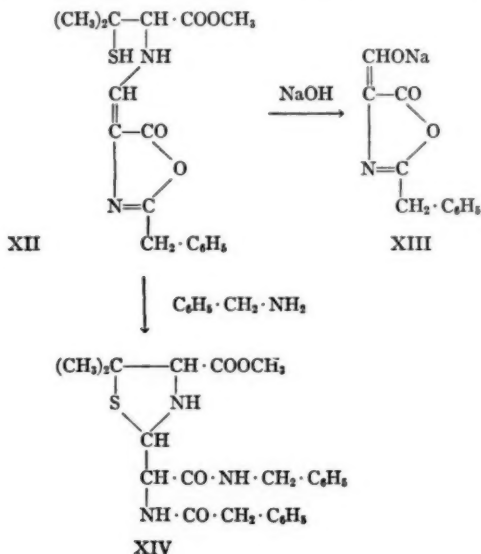
Formation.—When the sodium salts of the penicillins are treated with mercuric chloride in aqueous solution at room temperature, no immediate reaction occurs, but slow decarboxylation and cleavage into penicillamine and penilloaldehydes takes place which is complete after twenty-four hours (72). When the methyl-ester of benzylpenicillin is treated with mercuric chloride in

ethereal solution, a reaction product is formed in which the labile carboxyl group is retained. It possesses a low negative rotation (85, 168) and exhibits a strong absorption in the ultraviolet region at 3200 Å (169). It was shown to be the monomethylester of a dicarboxylic acid isomeric with methyl benzylpenicillin which was termed penicillenic acid (169).

Structure.—Condensation of D-penicillamine methylester and 2-benzyl-4-methoxymethylene oxazolone gave a product showing the same absorption spectrum as natural methyl benzylpenicillenate. Several pure synthetic products deriving from penicillamine and containing the grouping



showed a similar ultraviolet absorption. It was therefore considered that methyl benzylpenicillenate had the structure XII:



Further evidence for structure XII was obtained by cleavage of natural methyl benzylpenicillenate with sodium hydroxide which led to the formation of the sodium salt of 2-benzyl-4-hydroxy-methylene-oxazolone (XIII), and by treatment with benzylamine which gave the α -benzylamide of β -methyl-D-benzylpenicilloate (XIV) (169). The same substances were obtained from synthetic methyl benzylpenicillenate.

Syntheses.—(a) By condensation of 2-hydroxy- (or alkoxy-) methylene-4-alkyl (or aryl) oxazolones with penicillamine.

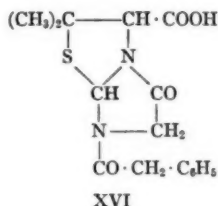
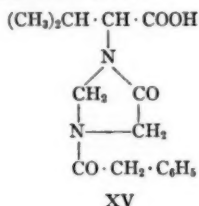
(b) By treatment of β -methyl penicilloates with azlactonising agents. Crystalline methyl amylpenicillenate was obtained by treatment of β -methyl-D-amylpenicilloate with benzoylchloride and pyridine (138).

PENILLONIC ACIDS

Formation.—When methyl benzylpenicillin is heated in xylene at 140° or refluxed in toluene in the presence of a small amount of iodine, it is transformed into a biologically inactive isomeric product, the methylester of an acid which was termed benzylpenillonic acid (134, 163). The same product is obtained from methyl benzylpenicillenate (138) by heating in toluene in the presence of a small amount of iodine. Methyl benzylpenillionate can also be obtained directly from methyl benzylpenicillin in 48 per cent yield by sublimation *in vacuo* (170), but not from methyl benzylpenicillenate; this suggests that penillonic acid is formed directly from penicillin as the result of an intramolecular rearrangement, and not indirectly by an intramolecular rearrangement of primarily formed penicillenic acid.

Reactions and structure.—Free benzylpenillonic acid can be obtained from the methylester by saponification with alkali. It is a monobasic acid, pK 3, possessing no basic centre. It is dextrorotatory (163). It is not decomposed by mercuric chloride and reacts with benzylamine to give the benzylamide of phenaceturic acid but only under drastic conditions (83). Phenaceturic acid was also obtained by heating methyl benzylpenillionate at 150° to 170° (169). Benzylpenillonic acid is stable to acids and alkali at room temperature, but on prolonged hydrolysis with 2.5*N* hydrochloric acid some phenylacetic acid is formed (95). With Raney nickel methyl benzylpenillonic acid is desulphurised to give methyl des-thiobenzylpenillionate, from which the free acid can be obtained

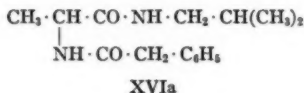
by warming with sodium hydroxide (106). On hydrolysis of des-thiobenzylpenillonic acid with concentrated hydrochloric acid glycine, *dl*-valine and formaldehyde (89 per cent yield) were obtained. On the basis of these findings the imidazolone structures XV for desthiobenzylpenillonic acid and XVI for benzylpenillonic acid were suggested (170).



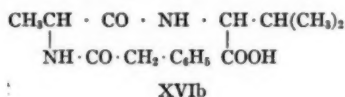
Synthesis.—Methyl benzylpenillionate was synthesised from synthetic methyl D-benzylpenicillenate by refluxing in toluene in the presence of a small amount of iodine (138).

HYDROGENOLYSIS OF BENZYLPENICILLIN

On treatment of sodium benzylpenicillin with Raney nickel three reaction products, a neutral and two acidic substances, have been obtained. (a) The neutral substance, $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_2$, was identified as the isobutylamide of *N*-phenylacetyl-L-(+)-alanine (XVIa) (157, 169, 171).



(b) One of the acidic substances, $\text{C}_{16}\text{H}_{22}\text{N}_2\text{O}_4$, isolated in form of its benzylamine salt, was shown to be identical with *N*(*N*-phenylacetyl-L-(+)alanyl)-D(-)-valine (XVIIb) (157).

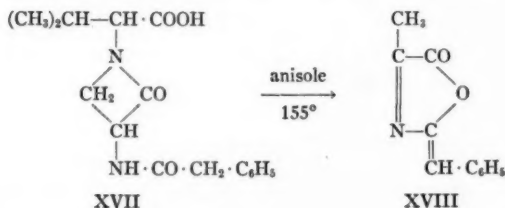


The isolation of XVIa and XVIIb established the configuration of the asymmetric carbon atom 6 in the penaldic acid moiety of the penicillins as the "natural" L-configuration. The isolation of the D(-)-valine derivative XVIIb is additional evidence for the unnatural configuration of the penicillamine moiety.

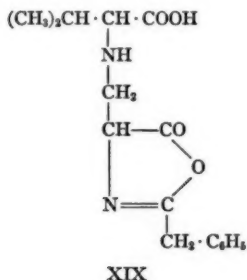
(c) The second acidic substance, $C_{16}H_{20}N_2O_4$, was termed desthiobenzylpenicillin.

Desthiobenzylpenicillin.—Desthiobenzylpenicillin was obtained in the form of its benzylamine salt (169) and by direct crystallisation of the free acid (157) after desulphurisation of sodium benzylpenicillin by heating with Raney nickel for ten to fifteen minutes at 100° , in aqueous solution (157) or in dioxane (92). The methyl-ester of desthiobenzylpenicillin was obtained by refluxing for five minutes a solution of methyl benzylpenicillin with Raney nickel in 50 per cent dioxane.

Desthiobenzylpenicillin was a monobasic acid, pK 3.6, showing no basic centre. The benzylamine salt has a low optical rotation, $([\alpha]_D^{20} + 9^\circ)$ (169). It is much more stable than benzylpenicillin to acid and alkali, being unaffected at pH 2 and 12 for at least several hours. On hydrolysis with 3*N* hydrochloric acid phenylacetic acid was obtained (157). On milder hydrolysis (with 0.5 *N* alcoholic hydrochloric acid at 60° to 75° for fifteen minutes or 0.1 *N* alkali on steam bath for fifteen minutes) D - α -desthiobenzylpenicilloic acid was obtained, identical with the product resulting from the hydrogenolysis with Raney nickel, followed by saponification, of α -ethyl- D - α -benzylpenicilloic acid. Desthiobenzylpenicillin is unaffected by benzylamine at room temperature, but on refluxing for three hours in dioxane it was converted into the α -benzylamide of D - α -benzylpenicilloic acid, identical with the sample obtained by hydrogenolysis with Raney nickel of the α -benzylamide of D - α -benzylpenicilloic acid (see above). Desthiobenzylpenicillin was stable towards boiling methanol, being recovered unchanged after refluxing for five hours (157), but in the presence of sodium methoxide it is converted into α -methyl- D - α -benzylpenicilloate after refluxing for twenty hours (172). On the basis of these facts, the β -lactam structure XVII was assigned to desthiobenzylpenicillin, the isomeric oxazolone structure (XIX) being excluded because of the stability to acid, alkali, methanol, and benzylamine.



Hydrogenation of desthiobenzylpenicillin with platinum oxide (92) or pressure hydrogenation with Raney nickel (173) gave desthiohexahydrobenzylpenicillin. Desthiobenzylpenicillin methylester, on refluxing in anisole at 155°, gave 2-benzylidene-4-methyl-oxazolone (XVIII) (174). The rearrangement of a β -lactam into an oxazolone is of interest in connection with the formation of penicillic acid from penicillin. When desthiobenzylpenicillin is treated with anhydrous hydrochloric acid in dioxane, the γ -isomer of desthiobenzylpenicilloic acid and D(-)valine are formed (92, 173). The formation of these products has been explained by intermediate formation of the unstable oxazolone (XIX) which undergoes mutarotation.



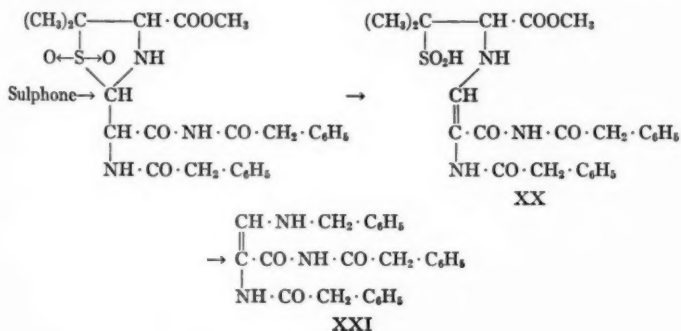
It was assumed that the oxazolone XIX was formed by rearrangement of the acid chloride of desthiobenzylpenicilloic acid produced by the action of anhydrous hydrochloric acid on desthiobenzylpenicillin. On treatment with benzylchloride and liquid ammonia desthiobenzylpenicillin was alkylated to give the N^7 -benzyl-D- α -desthiobenzylpenicilloic acid (174). Treatment of methyl desthiobenzylpenicillin with nitrosyl chloride under special conditions gave a crystalline N^7 -nitroso derivative (175).

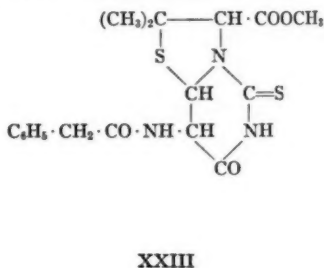
THE SULPHONE OF THE METHYLESTER OF BENZYLPENICILLIN

Isolation.—When the methylester of benzylpenicillin was treated with potassium permanganate in a solution of dioxane and phosphate buffer a crystalline compound was obtained in 85 per cent yield from chloroform extracts of the concentrated solution (92). It had the composition $\text{C}_{17}\text{H}_{20}\text{N}_2\text{O}_6\text{S}$, i.e., two oxygen atoms more than methyl benzylpenicillin, and its properties showed that it was the sulphone of methyl benzylpenicillin. The sulphone can

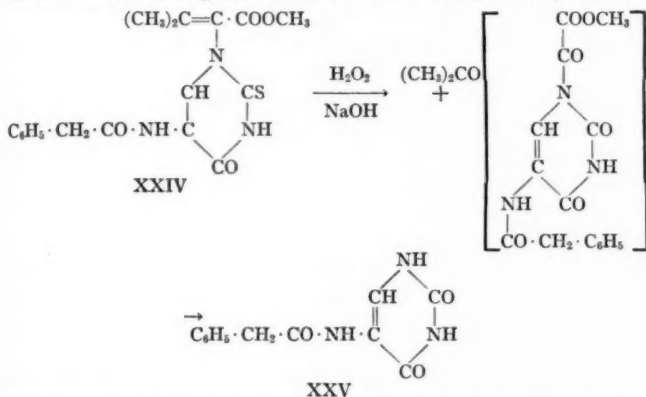
also be prepared by oxidation of methyl penicillin in 80 per cent glacial acetic acid (135).

Properties.—The methylester of the sulphone has no biological activity *in vitro* because of its low solubility. On saponification with 0.1 *N* sodium hydroxide and pyridine at 0°C., a product possessing an activity of about 40 units per mg. is obtained. The sulphone is active *in vivo*, protecting mice against *Diplococcus pneumoniae* Type I. It is about ten times less effective than sodium or methyl benzylpenicillin (173). With the exception of the esters the sulphone is the only derivative of the penicillins possessing biological activity. The sulphone of methyl benzylpenicillin is much more stable towards aqueous hydrochloric and glacial acetic acid than methyl benzylpenicillin; it did not show any change in rotation after standing for five hours in a mixture of equal parts of dioxane and 0.2 *N* HCl, and could be recovered unchanged after nineteen hours from solution in glacial acetic acid. On the other hand it was more reactive towards benzylamine and alkali (53). This shows the influence of the state of oxidation of the sulphur atom on the stability of the ring system in the penicillin molecule which is opened under the influence of hydrolytic agents. The reaction product of the sulphone of methyl penicillin with benzylamine was the benzylamide of α -phenylacetyl-amino- β -benzylamino-acrylic acid (XXI) (the benzylamine derivative of the benzylamide of benzylpenaldic acid) (173). The sulphone was stable in methanol for sixteen hours, but in the presence of 1 per cent *N*-ethylpiperidine it reacted rapidly and the 2,4-dinitrophenylhydrazone of methyl benzylpenaldate could be isolated (173). It was suggested that the reaction with benzylamine proceeded according to the scheme:



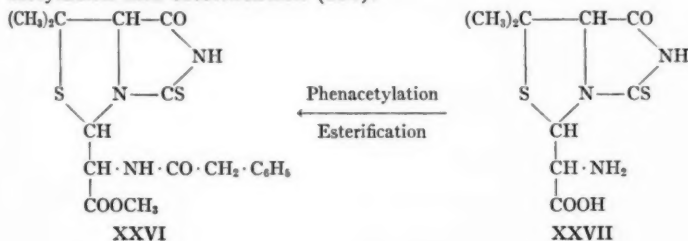


the thiohydantoin structure XXII was not compatible with the absence of a basic group and the resistance to mercuric chloride. The thiodihydouracil structure XXIII for the product became probable when by degradation with mercuric acetate (125)



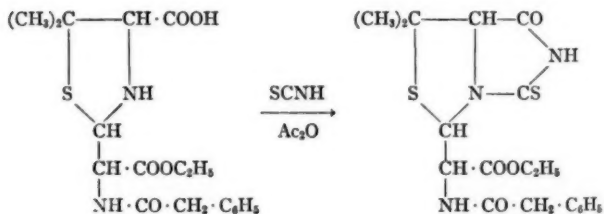
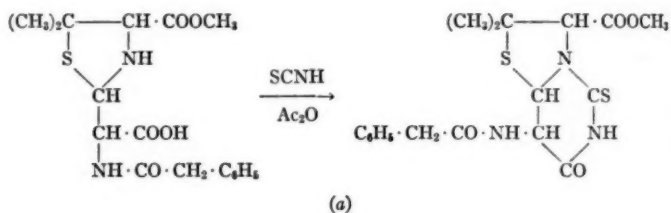
a product was obtained which gave acetone (181) and 5-phenylacetamidouracil (182) (XXV) on oxidation with alkaline hydrogen peroxide and had therefore structure XXIV (181, 182). Structure XXIII for the thiocyanate derivative of methyl penicillin was confirmed by direct synthesis (183).

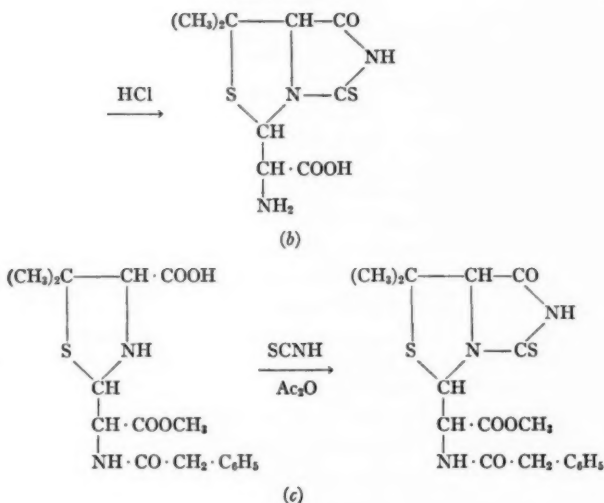
Rearrangement with alkali and acid.—On treatment with alkali in aqueous methanol the thiocyanate derivative of benzylpenicillin undergoes a rearrangement to XXVI. The isomerisation product has a high dextrorotation. The same rearrangement occurs on hydrolysis of the thiocyanate derivative with concentrated hydrochloric acid; simultaneously the phenylacetyl group is split off. The product of acid hydrolysis, which has structure XXVII (184), can be converted into the alkali isomerisation product by phenylacetylation and esterification (180).



The alkali isomerisation is not a purely intramolecular rearrangement. By carrying out the reaction in deuterio methyl alcohol, CD_3OD , it could be shown that methanol participated in it, as the reaction product contained a large proportion of stable deuterium (183).

Syntheses of thiocyanate derivative and its alkali isomerisation and acid hydrolysis products.—The thiocyanate derivative was synthesised from β -methyl-D- α -benzylpenicilloate by treatment with potassium thiocyanate and acetic anhydride containing a trace of sulphuric acid (183). Treatment of the γ -isomer of α -ethyl- β -methyl-D-benzylpenicilloate with ammonium thiocyanate in boiling acetic acid gave an isomer of the thiocyanate derivative (181). The acid hydrolysis product was synthesised from α -ethyl-D- γ -benzylpenicilloate by heating for ten minutes with ammonium thiocyanate and acetic anhydride. On hydrolysis of the reaction product a high melting isomer of the acid hydrolysis product was obtained which was converted to the low melting isomer by racemisation with acetic anhydride (184). The alkali isomerisation product was synthesised by treatment of α -methyl-D- α -benzylpenicilloate with potassium thiocyanate and acetic anhydride containing a trace of sulphuric acid at 5°C . for twelve hours (183). The syntheses are represented by the following schemes:





SEMI-ARTIFICIAL PENICILLINS BY FERMENTATION

Addition of phenylacetic acid to the culture medium was shown to increase the yield of penicillin in surface culture and submerged culture production (185). Incorporation of phenylacetic acid by the mold into the penicillin molecule was shown to take place on the basis of the observation that a mold strain which normally produced Δ^2 -penicillin yielded benzylpenicillin after addition of phenylacetic acid to the culture medium (186). By adding to the culture media substituted phenylacetamides and other precursors utilised by the mold new penicillins have been obtained, some of them in the crystalline state. Thus the sodium salts of *p*-methoxybenzyl, thienylacetyl, *p*-chlorobenzyl, *p*-nitrobenzyl (187), *p*-fluorobenzyl, *m*-fluorobenzyl, *o*-fluorobenzyl (188), *p*-bromobenzyl, phenoxyacetyl, tolylmercaptoacetyl, and *p*-iodobenzyl penicillin (189) have been obtained in the crystalline state by addition of the following precursors: *N*-(2-hydroxyethyl) *p*-methoxyphenylacetamide, *N*-(2-hydroxyethyl)- α -thienylacetamide, *p*-chlorophenylacetyl-DL-valine, *p*-nitrophenylacetyl-DL-valine, *N*-(2-hydroxyethyl)-*p*-fluorophenylacetamide, *N*-(2-hydroxyethyl)-*m*-fluorophenylacetamide, *N*-(2-hydroxyethyl)-*o*-fluorophenylacetamide, *p*-bromophenylbutyryl-DL-valine, *N*-(2-hy-

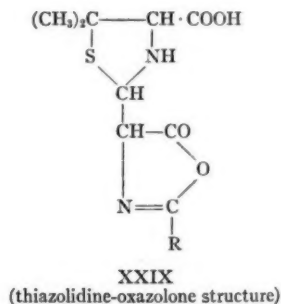
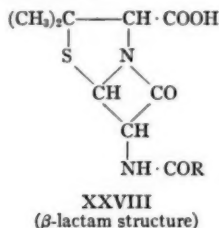
droxyethyl)-phenoxyacetamide, *p*-tolylmercaptoacetyl-DL-valine, and *N*-(2-hydroxyethyl)-*p*-iodophenylacetamide.

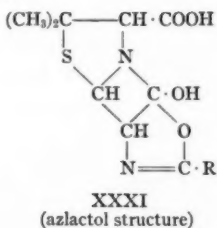
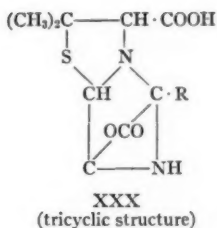
AZOPENICILLINS

Biologically active derivatives of *p*-hydroxybenzylpenicillin termed azopenicillins have been prepared by coupling in neutral solution with the diazonium salts of the following compounds: aniline, *p*-chloraniline, *p*-bromaniline, *p*-iodoaniline, *o*-nitraniline, *m*-nitraniline, *p*-nitraniline, 2-nitro-4-bromaniline, *p*-toluidine, 2,4-dimethylaniline, 2-naphthylamine, *p*-aminobenzoic acid, methyl-*o*-aminobenzoate, ethyl-*p*-aminobenzoate, sulphanilamide, benzidine, *p*-succinylphenylenediamine, *p*-acetylphenylenediamine, *p*-aminoacetophenone, hippuric acid, 1-aminoanthraquinone, and arsanilic acid (24). Only two azopenicillins, 3-(4-bromophenylazo)- and 3-(2-naphthylazo)-*p*-hydroxybenzylpenicillin, have been prepared in the pure state, in the form of their sodium salts, using partition chromatography on silica phosphate gel (24b), or salting out from cold water (24d). The azopenicillins act qualitatively against the same organisms as the natural penicillins, and quantitatively in the same order of magnitude. Their pharmacological properties have not yet been studied.

THE STRUCTURE OF THE PENICILLIN MOLECULE

Many formulae for the penicillin molecule can be devised by combination of the three basic constituents penicillamine, penilloaldehyde and carbon dioxide with the elimination of two molecules of water. A large number of these are in obvious disagreement with the properties of the penicillins and can be discarded, but four structures (XXVIII, XXIX, XXX and XXXI) have received serious consideration.



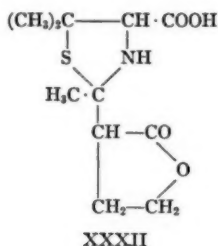


The β -lactam and thiazolidine structures were among the earliest suggestions (112, 115). It was pointed out (112) that the β -lactam structure, though it contained the unusual four-membered ring, was in better agreement with the properties of the penicillin molecule because it contained both nitrogen atoms in the nonbasic N-CO linkage, whereas the thiazolidine-oxazolone structure contained an imido group which would have been expected to show basic properties. The majority of workers favored at first the thiazolidine-oxazolone structure which contained two well known ring systems and was capable of explaining some of the chemical reactions of the penicillin molecule. The tricyclic structure (65, 159) contained the skeleton of penillic acid preformed and could thus explain the facile formation of this substance in acid medium, but failed to explain the formation of α -methyl penicilloates by the action of methanol on the penicillins and the nonbasicity of the two nitrogen atoms in the penicillin molecule. It was discarded when it was shown to be incompatible with the crystallographic x-ray data (190). The "azlactol" formula (191), a compromise between the β -lactam and the thiazolidine-oxazolone structure, offered no better explanation for the nonbasicity of the two nitrogen atoms in the penicillin molecule than the thiazolidine-oxazolone structure; in fact, its nitrogen, present in a carbinol-amine linkage, would have been expected to exhibit strongly basic properties. The "azlactol" structure was also shown to be incompatible with the crystallographic x-ray data.

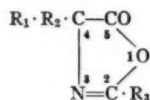
Chemical and physicochemical evidence in support of and against the thiazolidine-oxazolone and β -lactam structures was derived from the study of model compounds and of the degradation products of the penicillins. Many model thiazolidines, *N*-acylated and nonacylated, were synthesised. All thiazolidines with free imino groups readily formed salts and had basic groups with *pK* values of about 4 to 5 (65, 141), were oxidised by iodine to the S-S

compounds (94, 192), were rapidly decomposed by mercuric chloride (192), poisoned platinum and palladium hydrogenation catalysts (65, 193), and were oxidised by permanganate to the sulphonic acids; the *N*-acylated thiazolidines had no basic group (65, 141), were stable to iodine (94, 192) and gave sulphones on oxidation with permanganate (194). Thus it became clear that the penicillins behaved in every way like *N*-acylated thiazolidines with free imino-groups; their behavior in this respect was in agreement with the β -lactam structure and incompatible with the thiazolidine-oxazolone structure.

It was suggested that the basicity of the imino group in the thiazolidine-oxazolone structure was reduced by intraspatial influence of the carbonyl group in the oxazolone ring, acting as an electron attracting centre, like the CO group in a peptide linkage (195, 196). However, the basicity of the imino group in the model thiazolidine XXXII



prepared by condensation of penicillamine and α -aceto-butylolactone (197) was not significantly different from that of other thiazolidines, having a pK of 4.2 (198). Many oxazolones were synthesised. Oxazolones of the type



i.e., saturated in the 4-position, were much less stable in water than the penicillins (178, 199). With liquid ammonia 2-phenyl-4-benzyl-oxazolone and 2-phenyl-4-isopropylidene oxazolone reacted to give

the amides of the corresponding acylamino acids (200) whereas benzylpenicillin was stable under these conditions. These facts were difficult to reconcile with the presence of an oxazolone ring in the penicillin molecule.

The results of the degradation studies did not allow of a clear-cut decision in favour of one or the other of the two formulae. While the isolation of 2-benzyl-4-hydroxymethylene oxazolone from the methylester of benzylpenicillin appeared to be a strong argument in favour of the thiazolidine-oxazolone structure, the significance of this finding was largely offset by the isolation of desthiobenzylpenicillin, possessing the β -lactam structure. The degradation studies had shown that the penicillin molecule had the property of undergoing with great ease various intramolecular rearrangements, and the question to decide was which of the two ring systems found in the degradation products, the oxazolone ring or the β -lactam ring, was originally present in the penicillin molecule, and which was formed as the result of an intramolecular rearrangement. The isolation of the isobutylamide of *N*-phenylacetyl-L-(+)-alanine and of *N*(*N*-phenylacetyl-L-(+)-alanyl)D(-)-valine could be explained in the most straightforward manner on the basis of the β -lactam structure, but it could also be assumed that these products were formed as the result of an intramolecular rearrangement, and the same consideration applied to the interpretation of the results of study of the thiocyanate derivative of methylbenzylpenicillin.

The results of the analysis of thermochemical data [heats of combustion of model β -lactams, methyl benzylpenicillin, and dimethyl-D- γ -benzylpenicilloate (201, 202)] were compatible with the β -lactam structure though they did not eliminate the thiazolidine-oxazolone structure (203, 204). Theoretical argument to account for the reactivity of the N-CO linkage in β -lactams, particularly in the penicillin molecule, and electronic mechanisms for the penicillenic, penicillenic, and penillonic rearrangements have been advanced (205). The results of the extensive infrared studies, which cannot be discussed in detail, appeared to be incompatible with the thiazolidine-oxazolone structure, but were in agreement with the β -lactam structure when model compounds close enough to the structure of the penicillins became available.

The β -lactam structure was finally conclusively proved by

crystallographic x-ray studies on crystals of the rubidium and potassium salts of benzylpenicillin (206, 207, 208). By Fourier analyses of electron densities in three dimensions the bond distances between all atoms in the penicillin molecule were measured, the alkali metal and the sulphur atoms serving as "land-marks."

The whole molecule was thus mapped out and the existence of the four-membered ring demonstrated beyond doubt.

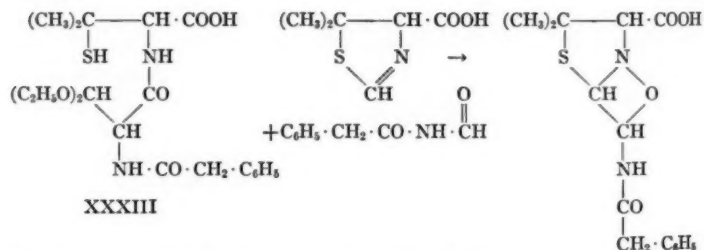
WORK TOWARDS THE SYNTHESIS OF PENICILLIN

Several routes for syntheses of penicillin have been explored. Of these the following may be mentioned:

(a) Ring closure of penicilloates. This has been attempted in many laboratories, using different derivatives of natural and synthetic D-penicilloic acids, such as α -esters, β -esters, α,β -esters, amides and N^4 acyl derivatives. Many azlactonizing agents have been used, among others phosphorus tribromide, different acid chlorides, anhydrides, and azides, ethyl and *n*-butyl magnesium bromide and phenylisocyanate. Elimination of nitrogen, carbon monoxide, aldehydes, hydrogen sulphide, and mercaptans from appropriate penicilloates was also attempted. In a few cases material possessing weak antibacterial activity was obtained, but there is no evidence that substances of the penicillin type were formed. Penicillenic acids are the main reaction products under these conditions.

(b) Cyclisation of *N*-penaldylpenicillamine. The di-acetal of *N*-benzylpenaldylpenicillamine (XXXIII) was prepared by condensation of the azide of benzylpenaldic acid diethylacetal with penicillamine (209). No biological activity has been reported from attempts to ring close this compound.

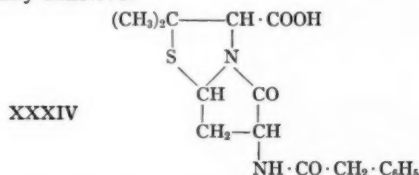
(c) Addition of ketenes to thiazolines (210) according to the scheme:



This route has so far not given successful results.

(d) Condensation of alkoxy-(or hydroxy-)methylene oxazolones with penicillamine. This route was originally designed for the synthesis of the thiazolidine-oxazolone structure (XXIX). Condensation of 2-benzyl-4-hydroxymethylene oxazolone with D-penicillamine (211) and of 2-styryl-4-ethoxymethylene oxazolone with D,L-penicillamine (212) gave a product possessing about 0.5 to 1 unit per mg. of antibacterial activity. Later products of the same order of biological activity were obtained by condensation of other 2-substituted alkoxy-methylene oxazolones with D-penicillamine. L-Penicillamine gave biologically inactive products (160). The product obtained by condensation of 2-benzyl-4-hydroxymethylene oxazolone with D-penicillamine acted against different types of bacteria in the same way as benzylpenicillin (213) and was destroyed by the enzyme penicillinase (214). When natural benzylpenicillin was added to the condensation product of 2-benzyl 4-methoxy-methylene oxazolone with D-penicillamine containing radioactive sulphur and isolated as the triethylamine salt the radioactivity was retained through a number of recrystallisations of the sodium salt and the benzylpenillic acid derived from it (215). All this evidence showed conclusively that benzylpenicillin had been synthesised. By using the counter-current distribution method of fractionation (23) the synthetic product was purified and a crystalline triethylamine salt prepared. This was identical in melting point, ultraviolet and infrared absorption spectra, refractive indices and specific rotation with the triethylamine salt of benzylpenicillin (216). It has not been possible, up to the present time, to increase the yield of synthetic benzylpenicillin.

A homologue of benzylpenicillin, the γ -lactam of homobenzylpenicilloic acid (XXXIV), has been synthesised (158) by condensation of the diethylacetal of homopenaldic acid with D-penicillamine followed by lactonization with phosphorous pentachloride and pyridine, or by fusion at 150°. Homopenaldic acid was prepared by ozonolysis of 2-phenylacetylamine, 4-pentenoic acid and converted into the diethylacetal. The γ -lactam was found to be biologically inactive.



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RUMINANT DIGESTION

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The topic of ruminant nutrition was last reviewed in this journal by Marston (1) and in the intervening eight years, in spite of, or more probably because of World War II, there has been an increased interest in the general biology of ruminants, particularly in the special problem of the digestive processes of these animals. It is pleasant to be able to report that an increasing number of physiologists, biochemists, microbiologists, and nutritionists realise that the ruminant offers not merely an intrinsically interesting field of investigation, but one which will yield results of primary importance to man, and one which has already emphasised certain aspects of intermediary metabolism and digestion which might otherwise have been overlooked through continued use of conventional laboratory animals.

The following reviews have appeared during the past eight years. McAnally & Phillipson (2) have written a general review of ruminant digestion; Goss (3) has discussed certain aspects of ruminant nutrition; Hastings (4) has discussed the microbiological aspects of ruminant digestion; Hungate (5), in a review of the microbiology of symbiotic decomposition of cellulose, has emphasised the fact that the utilisation of microbes for the digestion of cellulose is widespread in the animal kingdom; and within the past year two general reviews have appeared, Baker *et al.* (6), Owen (7). This review will be confined to recent studies on ruminant digestion, and other work will only be referred to in so far as it is related to the processes of digestion.

There are no cellulases in the digestive juices of mammals, and the breakdown of cellulose, whenever it occurs, is brought about

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by microorganisms, which obtain their energy for growth by the fermentation of this and related materials. This necessitates the provision of a chamber where the food is retained for a sufficient length of time, and under conditions suitable for the action of the microorganisms. The basic adaptation to the herbivorous mode of life is, therefore, an enlargement of a portion or portions of the alimentary canal. The most usual form of adaptation is the enlargement of the caecum and colon and this reaches its highest development in the Equidae. The ruminants have, on the other hand, in addition to an enlarged caecum and colon, a modified and capacious gastric system. This forms the main fermentation chamber and is well suited to the maintenance of a large and active population of microorganisms. In these animals the diet is subjected to two fermentations: one preceding and the other following the conventional mammalian digestive process. For this reason the ruminants, as a group, are anatomically better adapted than other mammals to the herbivorous mode of life, and digest a greater proportion of the crude fibre of the diet.

CHEMICAL CHANGES IN THE RUMEN DURING DIGESTION

It is desirable, in any investigation on ruminant digestion, to study the three ruminants most important to man, viz., the ox, sheep and goat. In practice this has not been done, and various groups have selected one or other of these types. Some have chosen the ox, but where a kinetic study of the processes of digestion is the main object, the problem of obtaining representative samples from an organ containing about 50 kilogrammes of partially digested herbage, and which is far from homogeneous, is not readily overcome as Pearson & Smith (8) have pointed out. The goat has not been widely used as an experimental animal. The sheep has the merit that representative samples of rumen fluid can be readily obtained, and has been widely used, particularly for kinetic studies.

Access to the rumen and abomasum can be obtained through a permanent fistula so that the changes occurring during digestion can be followed by removing samples at regular intervals. The original technique described by Colin (9) has been widely used, with only minor modifications, to create large open rumen fistulae in cattle; other devices have been invented to close the fistula when not in use (10, 11, 12). Sheep are too small for this method and Quin

et al. (13) introduced the use of ebonite canulae to overcome this difficulty. Ebonite is light, easily worked on a lathe, and is unaffected either by the microorganisms of the rumen, or by the digestive juices. Various types of canulae have been designed (13, 14) and the surgical procedures used for their insertion have been described. It is preferable to exteriorise the canula through a stab wound.

Saliva.—The saliva of the ruminant provides the basal medium for the rumen microorganisms, and it is well adapted both in composition and volume for this function. McDougall (15) has made a careful study of both mixed and parotid saliva of sheep and there was no marked difference in the composition of the two types. The concentration of inorganic phosphate was somewhat higher than that recorded by Watson (16) and the gland was found to concentrate blood inorganic phosphate some ten to twenty times. The alkali of the saliva is in the form of sodium bicarbonate. It was estimated gasometrically after a preliminary equilibration with alveolar air at room temperature (15), a procedure which was found necessary to correct for losses of carbon dioxide during collection and storage of samples. The mean of seven determinations was 237 volumes per cent carbon dioxide. The pH of the parotid saliva, measured at room temperature, was found to be 8.3; and on the basis of the Henderson-Hasselbach equation was calculated to be secreted at pH 8.1. The concentrations of sodium and potassium were similar to those of serum. McDonald (17) has shown that the nitrogen of the saliva is largely in the form of urea. The volume secreted by a single parotid gland in twenty-four hours varied between 930 ml. and 1840 ml.

The course of fermentation.—The rate at which fermentation proceeds in the rumen may be assessed either by the quantity of gas produced or the chemical changes that occur in the rumen fluid. Washburn & Brodie (18) determined the total quantities of methane excreted in half-hour periods after a feed of hay and observed that whereas 2 l. were excreted in the first half hour after feeding, the quantity rapidly declined during the subsequent twelve hours to nearly one tenth of that volume, although later the rate of excretion was steadier. Analysis of the proportions of methane to carbon dioxide in the gas in the rumen at intervals, both before and after feeding, showed that the ratio was not con-

stant: it was considerably smaller before than after feeding. Twice as much carbon dioxide as methane was evolved from the rumen during the eight hours following a feed of hay.

Cole *et al.* (12) estimated that the volume of rumen gas produced by cattle (fed on alfalfa hay) was in the region of 5 l. in the half hour before feeding; one hour after feeding the volume increased to over 20 l. The subsequent decline was rapid during the next three hours. The gases were aspirated from the rumen through a wide bore canula, and it was claimed that no losses occurred via the esophagus. Quin (19) studied sheep fed on green lucerne or lucerne hay and obtained similar results, although in these experiments maximum evolution of gas occurred within half an hour of feeding. Quin took no precautions to avoid loss of gas from the rumen by belching. Other gases reported in the rumen are oxygen, nitrogen, hydrogen, hydrogen sulphide, and carbon monoxide. Of these, the last three are present in traces only; oxygen rarely exceeds 1 per cent of the total and is found only during the later stages of digestion. Nitrogen on the other hand may attain considerable proportions during this stage and probably represents the residue of air entering via the esophagus (18, 20, 21).

It has been calculated (22) that when the pH of the rumen is 6.9 or over saliva flowing to the rumen will take up carbon dioxide owing to the high concentration present; at a lower pH, however, carbon dioxide will be evolved. Studies on the pH of the rumen liquor of the sheep show that it is seldom above neutrality (11, 23 to 26). The mere fact that a fistula or canula must be opened in order to obtain a sample means a loss of carbon dioxide, so that, in spite of subsequent precautions to avoid contact with the air, all values recorded are liable to be high.

After feeding the pH falls and this change coincides precisely with the production of volatile fatty acids (25). It is clear that part of the carbon dioxide must be produced by interaction of these acids with the bicarbonate of the saliva. The magnitude of the changes in acidity in the rumen of sheep varies with the diet; they are more abrupt following a feed containing starch or sugar than following a feed of hay. The acidity is greatest, however, when sheep graze young summer grass; then the average pH is approximately 6, in contrast to an average figure of 6.5 with animals that are stall fed. Similar changes in the pH of the rumen contents of

cattle can be accepted as indicative of acid production, for volatile fatty acids are known to be present (27).

Clear evidence of fermentation was obtained when individual carbohydrates were introduced in the rumen (28) and the results obtained were complementary to those changes noticed with normal diets. Cellulose, starch, and a variety of sugars, including cane sugar and glucose, were tested, and, with each, clear evidence of fermentation was obtained. With cellulose and starch, the increase in acidity only became obvious when the curve for volatile fatty acid production was compared with that obtained when neither food nor carbohydrate was given. Fermentation of cane sugar and glucose was rapid and marked by the transient appearance of lactic acid. One hundred grams of glucose placed in the rumen of a sheep, maintained on a mixed diet, disappeared within four hours; little if any glucose passed to the abomasum and the absorption of glucose as judged by the blood sugar was insignificant.

The only available evidence on the quantity of cellulose digested in the rumen is that obtained by the use of lignin as a marker, for lignin is sparingly, if at all, digestible (29, 30, 31) and is an integral part of the fodder. A change in the lignin to cellulose ratio can therefore be used as a basis on which to calculate the degree of digestion of cellulose. Hale *et al.* (32) have estimated by this method that 85 per cent of the digestible cellulose of hay disappears from the rumen of a cow within fourteen hours, while Gray (31), working with sheep fed on wheat straw and lucerne, showed that 70 per cent of the digestible cellulose was fermented in the rumen. These calculations are based on the lignin-cellulose ratio at a specific time and indicate only that the quantity of cellulose digested in the rumen is considerable.

The fate of hemicelluloses, an ill-defined group of substances, has barely been investigated. McAnally (29) reports that wheat straw hemicellulose rapidly disappears when enclosed in a silk bag and suspended in the rumen, while recently McNaught & Smith (33) have observed that L(+)-arabinose and D(+)-xylose, components of the hemicellulose fraction, can serve as a source of energy for rumen microorganisms. It is clear from the evidence discussed, that not merely cellulose, but all carbohydrates found in fodders are fermented by the rumen population.

The fermentation of carbohydrates by rumen microorganisms has been studied *in vitro* (19, 34, 35, 36) and in general the results obtained have tended to confirm those obtained by the more laborious *in vivo* method. The rate of gas formation was used by the Onderstepoort workers (19, 34) as an index of the ability of the rumen population to ferment a variety of carbohydrates. Elsdén (35, 36), using rumen contents diluted tenfold with a bicarbonate-saline solution, showed that the mixture of fatty acids produced from a variety of carbohydrates and related substances was qualitatively similar to that produced *in vivo*; but quantitatively the ratio of propionic acid to acetic acid was greater *in vitro*. However, it was possible to show that during the fermentation of glucose *in vivo* the ratio of propionic to acetic acid increased. The fermentation of dried grass *in vitro* yielded more acetic acid than propionic acid. McNaught & Smith (37) have drawn up a carbon balance sheet for *in vitro* fermentations of maltose by ox rumen liquor and have shown that most of the carbon could be accounted for as volatile acids, lactic acid and cell material.

Some of the energy of the food is liberated as heat during the fermentation (38) for the temperature of the rumen rises after feeding. It remains to be seen whether the known products of fermentation are produced in sufficient quantity to account for the changes in the diet during its passage through the rumen or whether other products of fermentation at present unsuspected are formed.

The identity of the acids present in the rumen remained in doubt until Elsdén (35, 39) developed a partition chromatogram which separated quantitatively mixtures of fatty acids. Acetic, propionic and butyric acids were always present, and traces of a higher acid were sometimes noted. Acetic acid was present in greatest amount, forming 55 to 75 per cent of the total. A similar mixture has been identified in the large intestine of a number of species (27, 40).

The total quantity of volatile fatty acid present in the reticulum and rumen of sheep fed on grass amounted to 64 gm., expressed as acetic acid (41), while the quantity present in the caecum was 5 gm. or less. In the rumen of the ox figures of 286 gm. and 371 gm. were found for two animals one and one-half and three years old respectively (27). These amounts are large enough to be interesting but give no indication of the daily turnover. It has been

shown that the amount of volatile fatty acid in the caecum and colon, in proportion to the body weight, is greatest in the horse, less in the pig, rat and rabbit, and least in the dog (27, 41). It is tempting, on this evidence, to suggest that the role played by alimentary microorganisms in these species decreases in this order.

THE FATE OF THE VOLATILE FATTY ACIDS

The quantity of volatile acid in the abomasum is small compared with the quantity present in the rumen (28). This is due to the absorption of acetic, propionic and butyric acids from the rumen reticulum and omasum, in spite of the fact that these chambers are lined with stratified squamous epithelium (42, 43, 44).

The proof of absorption rests upon the fact that blood draining the rumen carries a much higher concentration of volatile fatty acids than arterial or venous blood from any other part of the alimentary tract. In addition, when solutions of sodium acetate, propionate or butyrate, in approximately equimolar solutions, were introduced into the empty washed-out rumen, volatile acid was found in the blood draining the rumen in excess of that in arterial blood. The blood levels observed were such as to suggest that, under these conditions, acetate is the most rapidly absorbed and the size of molecule governs the rate of passage through the epithelium lining the rumen (44). Danielli *et al.* (45) found that the undissociated acid is absorbed at a greater rate than the anion and the order of absorption is reversed. Gray (46) studied the change in ratio of acetic to propionic acids in solutions introduced into the empty rumen of a sheep. No change was found when sodium salts only were present, but at a pH of 6.5 propionic acid disappeared more rapidly than acetic acid. Gray considers that more propionic acid is formed than the proportion of acids present in the rumen fluid suggests, and that the reason for the predominance of acetic acid is that propionic acid is absorbed more rapidly. If this is so then the ratio of propionic to acetic acids in the blood draining the rumen should be greater than it is in the rumen fluid.

The mixture of acids in the blood draining the normally filled rumen distills at a rate which suggests that the proportion of acetic acid is even greater in the blood than in the rumen (44). Analysis of the mixture of volatile acids in both the blood and the rumen by the partition chromatogram provides stronger evidence still that acetic acid is absorbed more rapidly than propionic acid from the

normally filled rumen (47). It remains to be seen whether sufficient volatile acid is carried by the lymph stream to make any significant difference.

The rapid absorption of free acid is a means whereby the products of fermentation are removed from the rumen so that the system is able to continue without embarrassment from an accumulation of metabolites or from any gross change in pH. In addition the ruminant obtains an immediate and continuous supply of easily metabolised substances from its food.

Measurements of the amount of volatile fatty acid in the blood draining the rumen of sheep fed on grass, together with measurements of the rate of blood flow, show that the quantity absorbed from the rumen and reticulum, expressed as acetic acid, is not less than 1 to 5 gm. per hour (42, 44). Volatile acid is also absorbed from the omasum, but even so a small amount passes to the abomasum. Volatile fatty acids are produced in and absorbed from the caecum, so that estimations of the quantity of volatile acids absorbed per day, based solely on rumen experiments, must be minimal.

Acetic acid is the main component of the volatile acid mixture, and there is now considerable evidence to show that this compound is both a source of energy and an important anabolic intermediary. Lusk (48) showed that feeding 3 gm. of acetic acid to a dog gave, within one hour of administration, a heat increment equivalent to 3.1 calories over and above the resting rate, while McManus *et al.* (49) showed that glycerol and glucose could be replaced by triacetin in the diet of growing rats without impairment of the rate of growth. The fact that the amount of acetic acid in the systemic circulation is small while relatively large amounts are formed in the blood draining the rumen (44) suggests that sheep too are capable of metabolising acetic acid. This is confirmed by the observation that acetic acid may be dripped into the rumen at the rate of 4 to 5 gm. per hour over periods of fifty hours without any significant increase in the amount excreted in the urine and with no change in the alkali reserve of the blood (50). Smyth (51) has shown that the cat utilises 5.4 to 7.0 millimoles acetic acid per kg. body weight per hour and that hepatectomy reduced this to 3.5 to 5.7 millimoles per kg. body weight per hour, indicating that the liver plays an important part in the metabolism of this compound [see also (52, 53, 54)]. Barcroft *et al.* (55) have shown that the

isolated perfused rabbit heart would remove acetate from the perfusion fluid at least as rapidly as glucose. Lorber *et al.* (56) have now confirmed and extended this. They have found that the respiratory carbon dioxide, produced by cats' hearts perfused with blood containing acetate labelled on the carboxyl group with C^{13} , was enriched with C^{13} .

The conception of acetic acid as an important anabolic intermediary has arisen as a result of the use of labelled acetate. Rittenberg & Bloch (57) have shown that the synthesis of long chain fatty acids probably occurs through the condensation of acetic acid units. It also appears that acetic acid is concerned in the formation of cholesterol (58). Lorber *et al.* (59) have shown that after administration of acetate labelled on both the methyl and carboxyl groups the glycogen of the liver is built up from glucose units in which all six positions are enriched with the isotope; whereas when enriched carbon dioxide was given as bicarbonate, only positions 3 and 4 of the glucose units proved to be labelled (60). This indicates that acetate can be converted directly to glucose.

The daily turnover of acetate in the rat has been estimated by Bloch & Rittenberg (61). They showed, with the aid of labelled acetate, that acetate is an effective acetylating agent for both *p*-aminobenzoic acid and sulphonamide. On the assumption that acetic acid is the only acetylating agent, they calculated, from the dilution of the labelled acetate fed, that 15 to 20 millimoles of acetate were formed per 100 gm. of rat per day. If this figure is correct, then acetate is a most important metabolic intermediary.

Thus the ruminant produces and absorbs a substance, acetic acid, which is usually considered to be a metabolite rather than a product of digestion. In addition, propionic acid is known to be converted to carbohydrate, while butyric acid behaves as other long chain fatty acids. There is therefore no reason to suppose that the volatile fatty acids are waste products of digestion; on the contrary, the large heat increment which follows feeding in the ruminant (62) may well be in part the result of their rapid metabolism.

MICROBIOLOGY OF THE RUMEN

It is essential that the population of the rumen as a whole be given at least as much attention as the study of the individual organisms. A technique has been developed (63) for counting the

microorganisms of the rumen, and while it is highly improbable that this procedure will give absolute figures for total counts, the method appears to give reproducible results, and for this reason it appears to be an adequate method for comparative purposes.

With this technique it was shown that, on a diet of wheat straw, the population was of the order of 5.8×10^8 bacteria per ml.; supplementing this diet with starch produced no significant change, whereas addition of urea as a source of nitrogen increased the population by 74 per cent. In the same way it was shown that addition of bone meal, as a source of phosphate, resulted in an increase of 34 per cent in total numbers of microorganisms. When urea was replaced by fish meal as a source of nitrogen the population increased to 1.9×10^9 cells per ml. (327 per cent). These experiments show that the nature of the diet conditions the size of the rumen population.

Harris & Mitchell (64) showed that on a diet deficient in nitrogen 17.8 per cent of the cellulose was digested; supplementing the diet with urea increased the digestibility to 38.7 per cent. This has not been confirmed (63, 65). Lardinois *et al.* (66) observed that supplementing the diet with urea resulted in an increased synthesis of the B group of vitamins. Molasses or starch, added to the diet of sheep, reduced the digestibility of cellulose (63, 65, 67, 68). These experiments suggest strongly that the composition of the diet affects the composition of the population. This hypothesis can only be tested by the development of specific methods for counting the different types making up the rumen population, and such methods depend on a thorough knowledge of the nature and properties of these organisms.

Changes in the composition of the rumen flora of sheep have been observed to accompany changes in the diet (19, 35, 69), and these changes have been correlated with changes in the chemical activity of the population. The rate of fermentation of glucose in the rumen of sheep, fed poor quality hay, was slow; when this hay was replaced by lucerne (19), or by clover hay (35), the ability to ferment glucose was markedly increased, and this was correlated with a change in population which could readily be observed microscopically. On any given diet the population appears to be remarkably constant, although the nature of the factors responsible for this constancy have not as yet been investigated.

The isolation in pure culture and study of the organisms responsible for the chemical changes in the rumen are most important but at the same time it must be emphasized that the analysis of the rumen population will not be achieved by the haphazard application of standard bacteriological procedures. Careful attention to the composition of the media and the conditions of growth is needed. Johnson *et al.* (70) counted rumen organisms by plating out on a simple medium and incubating aerobically; they obtained the astonishingly small figure of 6.5×10^6 organisms per ml. These figures show only the number of organisms which grow under the conditions provided, and as neither the medium nor the method of incubation resembled the conditions in the rumen, no significance can be attached to these results. Reliable viable counts of the rumen population can only be obtained by the use of specific cultural conditions, and it is certain that no one medium will be adequate for all organisms.

Two criteria must be fulfilled before an organism can be said to be a functional member of the rumen population: (a) the organism must perform a chemical reaction known to occur in the rumen; (b) the organism must be present in the rumen in numbers sufficient to perform this reaction. These criteria have been applied to few if any bacteria isolated from the rumen. Van der Wath (63) observed that in sheep fed on a starch-rich diet numerous iodophile cocci developed and congregated around disintegrating starch grains. This organism was isolated in pure culture and found to be a gram positive streptococcus which formed acid from glucose and starch. Unfortunately neither total nor viable counts were made and no physiological studies reported so that at the moment it is impossible to assess their real significance. Baker (71) has observed similar organisms in the caecum of the rat.

A yeast-like organism develops in the rumen of sheep when lucerne (19), mangolds (69), or a high quality clover hay is fed (35). Johnson *et al.* (70) made total counts and found 2.5×10^7 to 8.3×10^7 organisms per ml. rumen liquor. Quin (19) associated them with a particularly rapid type of fermentation. He classified the organism as a Schizosaccharomycete on the grounds that it had the general shape of a yeast and multiplied by binary fission. This is scarcely adequate information on which to classify an organism, and it is preferable to use the term "yeast-like" until such time as

the organism is isolated in pure culture. It may well not be a yeast for alcohol formation by it has never been detected in fermentations either *in vivo* or *in vitro* (35, 36), and the production of alcohol is typical of fermenting yeast.

The isolation in pure culture of anaerobic cellulose bacteria has exercised microbiologists for a very long time but it is only in recent years that unqualified success has been achieved. In the ruminant the breakdown of cellulose seems to be associated with iodophile bacteria which can be found in large numbers attached to the plant debris of the rumen contents (72 to 77). These organisms appear to be responsible for the digestion of cellulose as they are found in "enzyme cavities," on the fibrous portions of plant materials and also on pure cellulose. This information is very valuable and provides a morphological standard against which to compare organisms isolated in pure culture. Hungate has made a significant contribution to the technique of isolating anaerobic cellulose bacteria. He has isolated *Clostridium cellubioparus* (78) from the rumen of the ox, and *Micromonospora propionici* (79) from termite gut and from cultures of rumen protozoa; these, while of interest as cellulose-fermenting organisms, do not appear to be of much significance in the rumen. On the other hand the isolation of two apparently significant types of cellulose bacteria from the rumen of the ox has been recently reported (80). One, a gram negative iodophile coccus seems to be similar to the iodophile cocci referred to above; the other is a gram negative rod. The medium used contained, amongst other things, sterile rumen liquor and 0.5 per cent sodium bicarbonate, and was saturated with carbon dioxide. The importance of bicarbonate in the media has also been stressed by Vartiovaara *et al.* (81). This organism accounts for less than one per cent of the total population. No information on the nature of the products of fermentation is as yet available. This work is of such importance that its confirmation is needed.

The appearance of lactic acid as an intermediary in the decomposition of glucose by rumen microorganisms, both *in vivo* and *in vitro* (25, 28, 35), suggests on the one hand the presence of lactic acid bacteria and on the other the presence of lactic acid decomposing organisms. Van der Wath's streptococcus may be one of those responsible for the production of lactic acid. Two types of lactic acid decomposing bacteria have been found in the rumen

of sheep. Strains of propionic acid bacteria were isolated from cellulose and glucose enrichment cultures (35), and when yeast extract-lactate enrichments were made, with rumen liquor as inoculum, a weakly gram positive micrococcus which formed hydrogen, carbon dioxide, propionic and acetic acids was isolated (82). No true Propionibacteria were isolated by this procedure, possibly because the micrococcus grows very rapidly as compared with Propionibacteria. The status of both these organisms needs investigation, for while the occurrence of propionic acid in the rumen makes it reasonable to expect propionic acid forming bacteria, we have no knowledge of the numbers of either type present.

If hydrogen is produced at all in the rumen, it is in very small quantities; Lugg (83) in a very careful study found none at all. This is curious for hydrogen is a common end product of fermentation. It may be that hydrogen is formed but it is rapidly used by other bacteria present. So far no search has been made for hydrogen bacteria in the rumen, and such an investigation would be well worth the effort. Similarly we have no knowledge of the origin of methane in the rumen, but Barker's work (84, 85, 86) makes clear the type of reaction to look for and the cultural procedures to use.

The problem of rumen protozoa has often been discussed; there is now plenty of evidence in favour of the concept that they play no vital role and can be dispensed with without any loss to the host (63, 87, 88). It has been shown (63) that on starch-rich diets Entodinia predominate, and these ingest starch grains. It is claimed that the disintegration of starch is brought about by iodophile cocci residing in the protozoa. On cellulose-rich diets the Diplodinia predominate (63) and these have been observed to ingest particles of fibre, the digestion of which, it is claimed, is brought about by symbiotic bacteria. Hungate (89, 90) has raised clone cultures of a number of species of Diplodinia. They were all strict anaerobes and required cellulose and dried grass in the medium. The generation time was about twenty-four hours, and all contained a cellulase which could be obtained cell-free. It was not decided whether this cellulase was produced by the protozoa or by the symbiotic bacteria as claimed by van der Wath (63). *Entodinium caudatum* was also cultured by Hungate (90); this was found to require starch and could not use cellulose. The results of Hungate and van der Wath are complementary and represent a

pretty combination of the observational and the cultural approaches.

WATER-SOLUBLE VITAMINS IN THE RUMEN

The hypothesis that the microorganisms of the rumen synthesise the B group of vitamins (91) has received support from recent work. Pearson *et al.* (92, 93) and Winegar *et al.* (94) showed that lambs maintained on a diet deficient in nicotinic acid remained normal: synthesis of nicotinic acid was assumed to occur either in the rumen or in the tissues for the concentration in the blood and the amount excreted in the urine were normal.

McElroy & Goss (95) reported that rumen contents from a sheep fed for thirty days on a diet deficient in B complex contained greater amounts of thiamine, riboflavin, pantothenic acid, and vitamin B₆ (pyridoxine) than the food. Subsequently the same authors (96) reported that a fistula cow was maintained in good health for four and one-half months on a B complex deficient diet, during which period she calved successfully and reared her calf. The rumen contents were assayed for pantothenic acid and nicotinic acid; riboflavin and pantothenic acid were determined in the milk. The content of pantothenic acid and nicotinic acid in the rumen materials was greater than that of the diet; and the concentration of riboflavin and pantothenic acid in the milk was the same as that in milk from animals fed on a normal diet. A more complete account of these experiments was published later [McElroy & Goss (97 to 100)] in which it was shown that a synthesis of vitamin K, in addition to the B complex, occurs in the rumen. In the case of the lactating cow, the concentrations of thiamine, riboflavin, pyridoxine and pantothenic acid in the milk were normal. There was only one discrepancy between the rumen analyses on the sheep and the cow—thiamine. With sheep, McElroy & Goss (99) found that the rumen contents contained seventeen times as much thiamine as an equivalent weight of the diet; but in the case of the cow no such difference was observed, and the rumen contents contain the same amount of thiamine as the diet. The fact that the milk of the cow was normal in respect to thiamine can perhaps be explained by assuming that the animal was secreting the vitamin at the expense of its tissues. McElroy & Jukes (101) showed that the biotin content of the material from the rumen of the cow was greater than that of the diet.

Similar investigations have been made by Wegner *et al.* (102) with substantially the same results except that a synthesis of thiamine, as well as riboflavin, nicotinic acid, pantothenic acid, pyridoxine, and biotin was reported. The animal used was a Holstein heifer with a permanent rumen fistula. These workers further reported that supplementing the diet with thiamine resulted in an increased synthesis of the other members of the B complex with the possible exception of nicotinic acid. The increased synthesis brought about by thiamine was relatively small save in the case of biotin, where the addition of thiamine roughly trebled the biotin concentration. There was no evidence of thiamine destruction by the microorganisms of the rumen.

The newly developed methods of microbiological assay were used for the estimation of riboflavin, nicotinic acid, pantothenic acid, and biotin, and it is certain that these convenient techniques will be increasingly applied to the problem of vitamin synthesis in the rumen. This being so, it is most important that these methods, and for that matter any others, whether chemical or biological, are rigorously tested for their applicability to this particular material. So far this has not been done by any group of workers. No evidence was supplied that the extraction procedures used were quantitative, that the extractives from rumen contents had no adverse effect on the microbes used for the assays, and no figures for the recovery of known amounts of pure crystalline vitamin preparations added to rumen contents were given. Thus at this stage all that can be said is that when ruminants are fed on a diet deficient in the B complex, there occurs in the rumen, as a result of the activities of the microorganisms, a synthesis of the members of the B complex, with the possible exception of thiamine. That the increase observed is a true one and not an artifact seems certain for it is too great to be accounted for by a concentration of rumen liquor; further, the daily excretion of the B complex in the milk is greater than the total amount taken in the diet (97 to 100).

A second paper from the Wisconsin group [Wegner *et al.* (103)] reported that a synthesis of the B complex, with the possible exception of thiamine, occurs even when the animal is fed a normal diet. Supplementing the nitrogen of the diet by urea did not cause an increased synthesis of the B complex; on the contrary there was a decrease in the amount of riboflavin. The experiments were repeated on a calf with substantially the same results; in addition,

supplementing the diet with 200 mg. thiamine brought about an increase in the quantities of all the members of the B complex. Nicotinic acid was estimated both chemically and microbiologically and there was a discrepancy between the results so obtained.

Lardinois *et al.* (66) have studied the effect of the composition of the diet on the synthesis of the B complex in the rumen. Providing that readily available carbohydrate was present, the addition of urea increased the synthesis of riboflavin, nicotinic acid, biotin and pantothenic acid; pyridoxine and folic acid could not be well correlated with the composition of the diet. The amount of thiamine did not appear to be greater than that taken in with the diet, and its synthesis in the rumen of the cow is still unsettled. It is clear from these experiments that the presence of readily available carbohydrate is necessary for maximum production of the B complex by the microflora. This fits in well with the work of van der Wath (63) on the relation of the numbers of microorganisms in the rumen to the composition of the diet.

Hunt *et al.* (104, 105) studied the production of riboflavin in the rumen of cattle and found that the amount synthesised depended on the nature of the diet and on the time of sampling. Similarly with thiamine a sample taken from the rumen twelve hours after feeding contained less thiamine than the original diet, whereas a sample removed four hours after feeding contained more. Therefore the time of sampling is important, and perhaps it is the answer to the differences of opinion that exist on the synthesis of this vitamin. Certainly further studies on the time course of the synthesis of the members of the B complex should be made.

The synthesis of the B complex by bacteria in the alimentary canal is by no means limited to the ruminant and the whole subject has recently been reviewed by Najjar & Barrett (106). But this process is probably of greater importance to the ruminant than to other animals; many cattle and sheep spend a considerable portion of each year grazing on dried, parched, poor quality pastures, which are undoubtedly far from rich sources of this group of vitamins. It seems not unreasonable to suggest that survival on such a diet is in part due to the fact that the food is enriched with the B complex by the microorganisms of the rumen.

The ruminant resembles most other mammals that have been examined in that it can synthesise its own ascorbic acid. Knight *et al.* (107) have shown that when a massive dose (100 to 150 gm.)

of ascorbic acid is given either orally, or introduced directly into the rumen, it rapidly disappears with no increase in the concentration of ascorbic acid in the blood plasma or milk. Further, when rumen contents are incubated with ascorbic acid, the vitamin is completely destroyed.

THE FATE OF NITROGENOUS COMPOUNDS IN THE RUMEN

Little attention has been given to the fate of protein in the rumen, but, on the other hand, there have been numerous studies on the utilisation of simple nitrogenous compounds by the micro-organisms of the rumen. If it is assumed that the ruminant digests the microbial population produced in the fore stomach, then the very old observation that many types of microorganisms multiply on such simple sources of nitrogen as urea or ammonium salts implies that part at least of the protein of the diet of ruminants can be replaced by such compounds. Recent work has shown, unequivocally, that this is so (64, 92, 108, 109). It must be emphasised that this phenomenon is primarily one of microbial growth, and not the synthesis of free protein.

Urea has been used most extensively as a source of nitrogen and is rapidly hydrolysed in the rumen (110, 111). This observation is in keeping with the known widespread distribution of urease amongst the bacteria. The ammonia so formed is used by the organisms for growth, provided a suitable carbohydrate source is available (68, 112, 113). Pearson & Smith (113) studied different carbohydrates as energy sources for the rumen micro-organisms *in vitro* and found that maltose and starch were the best. Baker & Smith (114) showed that during the *in vitro* fermentation of maltose, protein "synthesis" was accompanied by a marked increase in the number of iodophile cocci. McDonald (17) found that the nitrogen in saliva was largely in the form of urea and pointed out that hydrolysis of urea in the rumen is a normal physiological process. This offers interesting possibilities concerning the circulation of nitrogen in ruminants.

Ammonia is formed when casein is added to rumen liquor *in vitro* (112, 113), and McDonald (17) has found that the ammonia concentration in the rumen of sheep increases when protein is added through a fistula and that the rate of increase is related to the solubility of the protein. There is therefore reason to suppose that at least some of the protein of the diet is utilised by bacteria.

The quantity of microbial protein passing to the abomasum is unknown although the extreme view put forward by Hastings (4) that the ruminant lives solely upon the fauna and flora of the rumen cannot be true since volatile acids are produced, absorbed and metabolised. Similarly the hypothesis that all the nitrogen of dietary protein is utilised for microbial growth is an exaggeration for McDonald (17) has shown conclusively that absorption of ammonia occurs from the rumen, and that ammonia is found in the abomasal digesta. There is no reason, therefore, to assume that there is an absolute "final common path" for nitrogenous or other dietary constituents.

Harris & Mitchell (64, 108) proved by beautifully designed experiments that urea was a useful source of nitrogen for lambs. They used a diet low in nitrogen, to which was added either urea or casein in various proportions. They found that the biological value of the dietary nitrogen decreased as the amount of urea increased. The importance of methionine in the nutrition of the sheep is emphasised by the work of Loosli & Harris (115) who found that urea supplemented with methionine was equivalent to linseed meal as a source of nitrogen, whereas urea alone or urea and sulphate were inferior. These results suggest that microbial protein is less valuable than casein and is deficient in methionine. Yeast protein also has a low biological value due to a deficiency in methionine (116). There is no direct estimation of the biological value to ruminants of the microbial protein of the rumen; figures are available, however, for the biological value of such protein in rats. Johnson *et al.* (68) separated ciliate-rich and bacteria-rich fractions from the normal rumen contents of sheep and cattle and, after repeated washing, fed the dried powder to rats and found values of 68 and 66 respectively. McNaught *et al.* (117) performed similar experiments by feeding large numbers of rats with the dried microbial substance obtained by incubating rumen microorganisms with maltose and urea. The biological value of the protein was 88. The digestibility of ciliate protein was greater than that of bacterial protein (68); on this score it was suggested that the ciliates of the rumen, by ingesting bacteria, rendered the microbial protein more digestible. Many reports are available that the ciliates disintegrate in the abomasum and the accuracy of this observation is easily confirmed by microscopic examination.

Thaysen (118) estimated the quantity of dry microbial substance

in samples of rumen fluid taken at varying intervals throughout the twenty-four hours and found an average figure of 404 mg. per 100 ml. If his assumption is correct that 100 l. of fluid pass to the abomasum in twenty-four hours then the cow obtains approximately 400 gm. of microbial substance equivalent to 180 gm. of protein daily. This figure is based only on the free microbial population. Baker *et al.* (6) have calculated that the cow receives about 75 gm. of microbial protein per twenty-four hours. These estimates show that the quantity of microbial protein passing to the abomasum is likely to be substantial.

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PHYSIOLOGICAL ASPECTS OF GENETICS¹

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The biological sciences appear to be in the midst of a period of unprecedented progress. An important salient in the advance is the general recognition of the significance of genes in the economy of the organism. The change is largely one in point of view. Many biologists have for a long time appreciated the basic nature of the gene and its role in development and function, but these relatively enlightened individuals have for the most part belonged to a small group of specialists that has tended to remain in isolation. The restraining fences are now being broken down with gratifying rapidity. Biochemists have begun to think in terms of genes because it has been demonstrated to them not only that the chemical reactions which make up living systems are under the fairly immediate supervision of these units of inheritance, but also that genetics provides a powerful tool with which a great deal can be learned about metabolic systems. In bacteriology, too, one sees the effects of an altered point of view toward genetics. Only a few years ago a bacteriologist who so much as raised the question of the existence of genes in bacteria was regarded as a renegade and heretic by his more orthodox fellowmen. It has now become acceptable to speak of bacterial genes; in fact, a recognized field of bacterial genetics has grown up almost over night (1).

Several general reviews of the field of genetics have appeared since Danforth's (2) contribution to this series (3 to 8). British wartime achievements in genetics have been summarized in the first number of the new *Journal Heredity* (9). In the same issue is found a most valuable bibliography of genetic papers published in Italy and Germany during the war years, 1939 to 1945 (10). The eleventh volume of the Cold Spring Harbor Symposia on Quantitative Biology entitled *Heredity and Variation in Microorganisms* (11) constitutes a series of vivid expressions of the new viewpoints in genetics applied to bacteria, viruses and other microorganisms. The first volume of a series of annual reports on Symposia of the

¹ This review covers the period from January 1, 1946 to July 15, 1947.

Society of Experimental Biology (British) has just appeared (11a). It contains nineteen significant and timely papers on nucleic acids.

The present reviewer has chosen for special consideration several topics in physiological or chemical genetics in which it appears that particularly significant developments are taking place. Limitations in space as well as in time, industry and insight of the author have precluded adequate consideration of other lines of work that may in the long run prove to be of even greater significance. In this latter category are recent additions to our knowledge of chromosome chemistry. It is not now clear to the reviewer just what are the interrelations of the genoprotein-T of Stern *et al.* (12), the chromosin of Mirsky & Pollister (13, 14) and the chromosomin of the Stedmans (15). Progress continues to be made in immunogenetics as evidenced by the reviews of Irwin (16, 17), additional work on inherited cellular antigens in pigeons and doves (18), and further interpretations of the data on *Rh* blood types in man (19, 20, 21). Additional experimental and theoretical consideration has been given to the phenomenon of position effect by Stern and his co-workers (22, 23), Gersh & Ephrussi (24), and by Goldschmidt (25). Among other advances in physiological genetics that cannot be adequately treated here are the determination of pigment-granule characteristics in a number of mouse coat-color types (26), the chromatographic fractionation of the red pigments of the *Drosophila* eye (27), the relation of the eye pigmentation to protein composition in *Ephesia* (28), studies on multiple alleles and modifier genes (29, 30, 31), and the effect of infrared radiation on the effectiveness of x-rays in producing mutations (32).

GENES AND BIOSYNTHESIS

Production and detection of biochemical mutations.—During the past half dozen years many mutant strains of microorganisms with altered growth-factor requirements have been studied. The frequency with which these arise may be greatly increased by treatment with ionizing radiation, ultraviolet radiation, and certain chemicals. The method of producing and detecting growth-factor mutants in *Neurospora* has been described in detail by Beadle & Tatum (33). It consists in testing individually numerous strains for ability to grow on a minimal medium. Essentially the same method has been used extensively by Fries (34) for detecting mu-

tant strains in the Ascomycete *Ophiostoma*. Recently Fries (35) has developed a technique of filtering out and discarding the descendant mycelia of spores that germinate and grow on basal medium. In this way he has increased the frequency of the mutant strains among tested individuals by a factor of approximately eight. Because of the strong tendency to heterocaryon formation in *Neurospora* it seems unlikely that the Fries technique can be applied without modification in this organism. Lederberg & Tatum (36) have devised a procedure for bacteria by which nonmutant individuals able to grow on the basal medium are allowed to produce colonies on an unsupplemented agar medium. After the locations of these are noted, mutant individuals are "developed" by pouring over the culture plate an agar medium with suitable additions. This method should be applicable to any colonial type microorganism. The use of colonial type mutants of filamentous fungi should make its use practicable in these organisms.

In the case of mutant strains of microorganisms that are capable of growth on a medium on which the original strain fails to grow, the classical enrichment technique of bacteriology can be used. This permits the examination of astronomical numbers of individuals. It has been applied to the measurement of mutation frequencies in bacteria (1), and in the study of back-mutation in *Neurospora* (37, 38). If a method comparable in efficiency with the enrichment technique could be devised for mutants with increased growth factor requirements, progress in chemical genetics could be enormously accelerated.

Genes controlling vitamin synthesis.—Numerous instances of inherited inability of *Neurospora* strains to synthesize specific vitamins of the B group have been reported (5). Tatum & Bell (39) have studied four mutant strains of this fungus that require thiamin or a related compound for growth. Each of these differs from wild type in a single gene. In one strain thiazole synthesis is blocked and in another the mold is unable to couple the thiazole and pyrimidine halves of the thiamin molecule. Two other mutants appear to be of a more complex nature. Monomethylaminoethanol has been shown by Horowitz (40) to be a normal precursor of choline. One mutant of the *Neurospora* that cannot make choline accumulates it while another is able to use it in place of choline. Mitchell & Houlahan (41) have investigated a riboflavinless *Neurospora* mutant strain that grows normally without exogenous

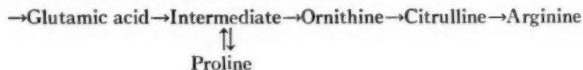
riboflavin at 25° C. and lower temperatures but gives little or no growth in the absence of this vitamin when grown at 28° C. or a higher temperature. Three *Neurospora* mutant strains that require nicotinic acid or a related compound have been reported by Bonner & Beadle (42). When grown under appropriate conditions, one of these mutants accumulates a precursor that can be used by another. This precursor of nicotinic acid is a pyridine compound but has not yet been completely identified. Recently Beadle, Mitchell & Nyc (43) have shown that one of the "nicotinicless" mutants grows when supplied indole, tryptophane, or kynurenine. The study of this mutant strain gives strong support to the hypothesis, based on work with mammals, that nicotinic acid is normally formed from tryptophane. The *Neurospora* evidence suggests that kynurenine is a normal intermediate in this process.

Numerous vitamin-requiring mutants of *Ophiostoma* have been obtained and studied by Fries (35). There are interesting differences in the relative frequencies of various types of mutants in *Neurospora* and *Ophiostoma* but the significance of these is not understood.

In connection with attempts to produce mutant strains of *Penicillium notatum* with increased penicillin-producing abilities, a number of growth-factor mutants were isolated (44). Many of these require specific vitamins for growth. Since this species has no perfect stage, inheritance studies by classical methods cannot be made.

Lindegren & Lindegren (45) have recently made crosses between strains of yeasts (*Saccharomyces*) differing in ability to synthesize certain vitamins of the B group. It is found that ability to synthesize pantothenic acid is differentiated by a single gene from inability to synthesize this vitamin. Similar relations are found between genes and ability to grow without exogenous thiamin, pyridoxin or *p*-aminobenzoic acid.

Biosynthesis of amino acids.—Examination of the properties of *Neurospora* and *Penicillium* mutants requiring arginine or a related compound for growth has prompted Bonner (44, 46) to suggest that glutamic acid and proline may be related to ornithine synthesis according to the following scheme:



Six types of mutant strains are known. In each, one of the six reactions indicated in the scheme is assumed to be blocked.

A mutant strain of *Neurospora* that has been difficult to understand in terms of a single blocked reaction is one that requires for growth the amino acids isoleucine and valine. A recent reinvestigation of this mutant by Bonner (47) indicates that the conversion of the keto acid analogue of isoleucine to isoleucine is blocked and that α -keto- β -methyl-*n*-valeric acid accumulated as a result of the interrupted reaction secondarily interferes with a reaction by which the α -ketoisovaleric acid is converted to valine.

A common class of mutants in *Neurospora* (48), *Ophiostoma* (49), *Penicillium* (44), and the bacterium *Escherichia coli* (50, 51, 52) is that in which sulfur metabolism is interfered with in one way or another. In some strains sulfate cannot be reduced to sulfite, in others any of a number of forms of organic sulfur can be used, and in still others, specific sulfur-containing amino acids must be supplied for normal growth. In studying the growth requirements of certain mutant strains in this category in *Neurospora*, Horowitz (48) has recently suggested that *Neurospora* synthesizes methionine as follows:



Cystathionine has been isolated from the mutant strain that cannot cleave it to form homocysteine. There is little doubt that it is the compound by which sulfur is transferred from a three carbon to a four carbon amino acid.

Other gene-directed syntheses.—Mitchell & Houlahan (53) have analyzed a number of purine-requiring mutant strains of *Neurospora*. Seven genetically distinct types were identified. Inosine is a precursor of adenosine and is itself presumed to be formed from a hypothetical precursor by a gene-controlled reaction. The postulated precursor apparently is not accumulated by the mutant strain that cannot convert it to inosine but is instead irreversibly converted to a purple pigment. Mutants in which purine metabolism is defective are relatively frequent in *Penicillium* (44), and in *Ophiostoma* (54). The studies of Fries on purine-requiring mutants of *Ophiostoma* agree with those of Mitchell & Houlahan in indicating inosine as a precursor of adenosine. A number of pyrimidine-requiring mutants have been found in both *Ophiostoma* (54) and *Neurospora* (5).

Emerson & Cushing (55) and Emerson (56) have reported two mutant strains of *Neurospora*, one that tolerates sulfonamide concentrations that would completely prevent growth of the original wild type, and another that actually requires a sulfonamide for growth. While studies of these strains have not as yet revealed the precise role of sulfonamides in cellular metabolism they seem to indicate quite clearly that these compounds do not act merely to displace *p*-aminobenzoic acid from the site in which it is needed for normal functioning of the cell.

These several examples of gene-directed chemical reactions add substantially to those previously known (5) and serve further to support the view that most of the chemical reactions by which organisms develop and function are under the fairly immediate supervision of genes.

MECHANISM OF GENE ACTION

The view that some genes act by controlling enzyme specificities is not new, but experimental evidence supporting it has not been impressive until relatively recently (5). Within the past several years many genetic traits have been studied in which primary gene action appears to be the control of specific chemical reactions. In a few instances such control can be shown to be through the mediation of enzymes. Mitchell & Houlahan (53) have shown that in a specific one-gene mutant form of *Neurospora* the ability to aminate inosine to give rise to adenosine has been lost. McElroy & Mitchell (57) found no detectable difference in the adenosine deaminase of the mutant and its wild type counterpart. It is possible that this deaminase is not the enzyme that catalyzes the reverse reaction, or that inosine amination is not the reaction primarily involved in the mutant strain.

Ginsburg & Kaplan (58) have presented evidence that a skin oxidase present in wild-type mice is deficient in strains homozygous for mutant alleles of the coat color gene *C*.

A consideration of cases in which gene mutations are correlated with specific chemical reactions had led to the formulation of the hypothesis that primary gene action in general lies in directing the synthesis of specific proteins and that genes bear a one-to-one relation to such proteins (5). This would mean that enzymes directing specific chemical reactions should bear such a relation to the genes from which their specificities are copied. Delbrück [discussion fol-

lowing paper by Bonner (46)] has raised the question as to whether the experimental data on which this hypothesis is based have not been automatically selected to favor it. If a gene were to control in a primary way the specificities of say three enzymes, an organism carrying only inactive mutant forms of this gene would fail in three corresponding reactions and, unless three reaction products were supplied to it from an outside source, it would have a small chance of surviving. In an organism like *Neurospora*, mutants of this kind would be likely to be lethal in practice because in general the three necessary reaction products would not be likely to be supplied under the right conditions. Even if such mutants were saved they would be difficult to analyze by the technique routinely used and would be likely to be put aside. There is no doubt that this objection is well taken. On the other hand, if one gene could direct the specificity of more than one enzyme, one might expect occasionally to find single gene mutants with double growth-factor requirements that could not be interpreted on the one-to-one hypothesis. The only clear cut mutant of this type so far analyzed is that requiring both isoleucine and valine for growth. Bonner (47) has shown that a reasonable explanation of it is possible in terms of one blocked reaction and hence one specific enzyme defect. It is clear that additional experimental evidence, possibly different in kind from that now available, is necessary before any decision can be made as to the generality of the one-to-one hypothesis.

The precise manner in which genes transfer their specificities to nongenic proteins remains almost a complete unknown. The answer to this question will almost certainly be essentially the same as the answer to the questions of how specific proteins in general are synthesized and how genes themselves are duplicated. Wrinch (59) has recently addressed herself to this general problem.

BACTERIAL GENETICS

It has already been pointed out that bacterial genetics has only just become a socially acceptable field of interest. The extent to which the genetic viewpoint has affected bacteriology can be appreciated by reading Luria's recent valuable and stimulating review *Recent Advances in Bacterial Genetics* (1). While there are no compelling reasons for doubting that bacteria have genes essentially similar to those of higher organisms or that there exists some precise mechanism for distributing these hereditary units to daughter

cells, there are a number of reasons why bacterial genetics appears superficially different from that of higher plants and animals. In the first place, observations are usually made on very large populations of bacteria, not on individual organisms. Such populations, whether they consist of colonies on a solid medium descended from single cells or suspensions of cells in a liquid medium, are usually of enormous size in terms of number of cells. With gene mutation rates of the same order as found in higher organisms, these populations will almost always be mixtures of diverse types, the proportions of the different types being determined by chance, relative mutation rates, and by selection pressures. Unlike higher multicellular organisms in which the germ line is differentiated early in development of the individual, any cell in a bacterial population is capable of continuing the line. Finally, although there is now suggestive evidence of some type of fusion process or sexual reproduction in one strain of *Escherichia coli* (60, 61), there has not been known in bacteria a recognizable and experimentally controllable sexual process. Since in classical genetics the gene is a unit of inheritance experimentally defined in terms of sexual reproduction, bacterial genes have had to be investigated by indirect methods. In view of these considerations it is perhaps not remarkable that geneticists and bacteriologists have heretofore had little influence on one another.

The physical basis of inheritance in bacteria.—As a result of the remarkable work of Robinow (62) and others (63), it has become clear that at least certain of the rod-shaped bacteria have well defined chromosome-like structures that go through regular changes during the bacterial division cycle. It seems almost certain that these structures provide the physical basis of orderly gene distribution to daughter cells during fission. In view of these findings, it is not difficult to believe that such structures exist in all bacteria.

Parallelism of genetic phenomena in bacteria and higher organisms.—One of the indirect methods that has been resorted to in the study of bacterial genetics is that of mutation frequencies. If bacteria have genes like higher organisms that undergo random mutation at measureable rates, it should be possible to analyze bacterial populations in terms of such mutation rates. It is difficult to measure mutation rates in bacteria directly because the nature of any given individual must be inferred from the colony (population) it gives rise to, because mutation rates of individual genes are likely

to be so low as to require an inordinate amount of work to measure them, and because the final equilibrium established in a bacterial population is almost always strongly influenced by selection pressures. In *Serratia marcescens*, color variations are frequent. Bunting (64) has shown that if populations of this bacterium are maintained in the logarithmic phase of growth under suitable conditions, there are no selection differentials among different color types and the equilibrium is dependent solely on relative mutation rates. Since these rates are high, they can be measured directly. From the final equilibrium established between red and pink types kept growing logarithmically the relative forward and back mutation rates for the red-to-pink change can be determined. The rate at which the final equilibrium is established is a measure of the absolute rates. The actual rates for the red-to-pink change and the reverse are 10^{-4} and 3×10^{-3} per bacterium per cell generation. The hypothesis that color in this bacterium is gene-determined and that the genes concerned show random mutation at definite rates is clearly consistent with the facts. Braun's analysis (65) of rough-smooth variation in *Brucella abortus* shows that the phenomena concerned are capable of being accounted for in terms of gene mutations plus selection pressures.

In particular instances of bacterial variation the question has often arisen as to whether the change observed was a result of an environmentally induced modification or due to spontaneous and random "mutation." To distinguish between these possibilities, Luria & Delbrück (118) have developed the so-called "fluctuation" test. This depends on the assumption that if a given variation is environmentally induced, the proportion of modified cells will be statistically constant from sample to sample whether these samples come from one or several clones. If, on the other hand, the change occurs spontaneously and at random, the frequency of modified cells will vary greatly from clone to clone depending on whether or not a mutation happened to occur early in clonal development. It is also possible by means of the Luria-Delbrück method to estimate mutation rates (1). This clonal grouping method has been applied to phage-resistance in *Escherichia coli* (1), to resistance to penicillin (1), and to sulfonamide in *Staphylococcus* (66), to resistance to radiation sterilization in *E. coli* (67), to histidine independence in *E. coli* (38), and to uracil independence in *Clostridium* (68). In all instances the cellular modifications responsible for these changes

were randomly distributed suggesting that they could have resulted from gene mutation. In all of these studies cultural conditions were purposely such as favor the selection of mutant types.

Witkin (67) has recently reported an interesting mutant type in *Escherichia coli*. As compared with the original, this mutant is much more resistant to sterilization by x-rays or ultraviolet. Its susceptibility to x-ray or ultraviolet induced "gene" mutations, however, remains unchanged (69).

Following the demonstration that genes concerned with the synthesis of vitamins, amino acids and other metabolites could be induced to mutate with x-rays and ultraviolet radiation, similar types of mutants were looked for in bacteria by Roepke *et al.* (119), Gray & Tatum (120), Tatum (70), Burkholder & Giles (71) and others. It is clear that essentially the same metabolic systems exist in *E. coli* as in the fungi *Neurospora* and *Ophiostoma*. For example, Lampen and his co-workers (50, 51, 52) have shown that mutant strains of this bacterium with altered sulfur metabolism parallel closely mutants found in *Neurospora* (48) and in *Ophiostoma* (49). Similarly, the ornithine cycle made evident in *E. coli* through the study of mutant strains (70) is similar to that in *Neurospora*. Roepke (70) has shown that tryptophane synthesis in *E. coli* occurs in a manner similar to that in *Neurospora*. Tatum (70) cites several other examples of similar striking parallels. Since the various syntheses referred to can be shown by direct genetic methods to be gene-controlled in *Neurospora* and *Ophiostoma*, it seems almost inconceivable that the agents which direct the same chemical reactions in bacteria and show the same properties of mutation are not genes with essentially the properties of those of higher organisms.

Delayed appearance of induced mutants.—As already pointed out, phage-susceptible bacteria may spontaneously change to phage-resistant types by a gene-mutation-like process. Demerec (72) and Demerec & Latarjet (69) have shown that the frequency of such mutations in *Escherichia coli* can be greatly increased with x-ray or ultraviolet treatment of bacteria. While making quantitative studies of the induction of such mutations, a phenomenon has been observed that may well contribute in an important way to an understanding of the division mechanism in bacteria. This is the phenomenon of the delayed appearance of induced mutations (69). Following raying in either the resting or growing condition, some induced phage-resistant mutants make

their appearance in the treated cell generation, i.e., without division of the bacteria, but the majority do not show up until one or more divisions have ensued. Only after about thirteen cell generations does the observed mutation rate return to the spontaneous level.

As Demerec & Latarjet (69) suggest, this result might be expected if the rayed bacterial population were heterogeneous with respect to the number of sets of hereditary units, i.e., were irregularly polyploid. Indeed Peshkoff's observations on *Caryophanon latum* (63) suggest that such a situation may exist in this bacterium. On the other hand, the expression of mutant changes may require the exhaustion of some substrate, and the time required for this may vary among individuals (69). Still another possibility is that the mutations may result indirectly through effects on the cytoplasm which may require several generations to disappear. In this connection a most interesting and significant discovery, made by Stone, Wyss & Haas (73), is that mutations for penicillin and streptomycin resistance are induced in *Staphylococcus aureus* by x-raying the medium in which the bacteria are subsequently placed. Here it is evident that the effect is indirect, possibly through the production of mutagenic substances in the medium. If such substances can be produced in the external medium it seems most likely that they would also be produced in the cytoplasm of a rayed cell and would result in delayed mutations such as Demerec & Latarjet have reported (69).

Possibility of sexual phenomena in bacteria.—The existence of some form of sexual reproduction in bacteria has been suggested many times. In fact morphological and experimental evidence have been brought forward in a number of instances [see review by Luria (1) for discussion and references], but to most bacteriologists and others this evidence has not seemed convincing. Sherman & Winge (74) appear to be the first workers to have used a genetic method in attempting to see if bacteria underwent any type of fusion and separation by which characters could be recombined. These experiments, made with the *coli-aerogenes* group and making use of the ability to utilize specific substrates as markers, gave inconclusive results because of instability of the parental lines. A similar approach to the problem was made by Gowen & Lincoln (75) using *Phytomonas stewartii* as test material. No evidence of the occurrence of a heterozygous phase or of recombination of characters was obtained.

Using growth-factor-requiring strains of *E. coli*, Lederberg & Tatum (60, 61) have recently obtained convincing evidence of fusion and segregation. Since the frequency of recombination types is low (of the order of 10^{-7}), it is necessary to use double or multiple mutant types as parents to exclude the possibility of reverse mutation as an explanation of the appearance of types having fewer mutant traits than either parent. The results appear to indicate true segregation. In fact, characters segregate in a nonrandom fashion indicative of a linkage mechanism of some sort (1). These findings are of revolutionary importance for they modify basic concepts regarding the biology of bacteria, particularly mechanisms of evolution, in these relatively simple organisms. Independent confirmation of the Lederberg-Tatum phenomenon is eagerly awaited, not because of any question as to the facts, but because of the great importance of the result and because of a natural question as to how widespread among bacteria the process is. In this connection it is of interest that Lederberg [unpublished result, cited by Luria (1)] and Luria (1) are unable to obtain similar results with another strain of *E. coli*.

GENETICS OF VIRUSES

Thirty years ago Troland wrote with remarkable insight about the interrelations of genes, enzymes and the then newly discovered viruses. It is unfortunate that he did not live to learn how prophetic his words were. One of the most fascinating recent developments in genetics is the demonstration by Burnet (121), Luria (1), Delbrück (76, 77), Hershey (78, 79), and others that bacterial viruses are mutable and that their mutation characteristics can best be accounted for on the assumption that an individual virus particle carries several gene-like mutation sites. Related viruses that attack the bacterium *E. coli*, strain B, vary in morphology as determined by electron microscopy, in antigenic properties, in host specificity and in plaque (clear area produced by lysis of bacteria on a Petri dish culture) characteristics. Spontaneous mutations in host specificity and in plaque characters have been observed and studied by several investigators [see Delbrück (76) and Hershey (78) for recent reviews]. Hershey, for example, has shown that the mutation from slow-lysing to rapid-lysing types, accompanied by changes in plaque characters, occurs with a frequency of once per approxi-

mately 10^3 virus duplications. The reverse mutation is much less frequent, occurring about one time per 10^8 duplications.

By the methods that he calls pattern analysis and rate analysis of mutant changes, Hershey (78, 79) has shown that lysis rate (detectable as a plaque character) and host range are independently mutating characters. It is found that the virus designated T2H, which has a certain host range and is a slow lyser, mutates spontaneously and irreversibly to one or another of two types with host ranges different from the original and from each other. All three host range types mutate reversibly to rapid lysers. A mutational change in lysis rate in the original T2H has no effect on the tendency of the virus to give host-range mutations. The rate of one of the two host-range mutations is identical in slow and rapid lysing strains. Essentially similar arguments are used to show that there are two independently mutable host-range factors and at least three independent sites influencing lysis characteristics.

Recombination of characters.—If bacteria are infected with a mixture of viruses not closely related as judged by morphology and serological properties, only one of the two types is multiplied in a given bacterium, a phenomenon called mutual exclusion. The yield of the successful type may be reduced markedly by the unsuccessful competitor [see Delbrück (76) for review]. If, however, two virus strains used in mixed infection are closely related, both may multiply within a single bacterium (76, 78). Delbrück & Bailey (77) observed that if the related types that multiply in a single bacterium differ in lysis characteristics (detectable also as plaque differences), new types may emerge, recombining characters of the virus strains with which the mixed infection was made. Thus if a bacterium is infected with $T6r$ and $T4r^+$ (r and r^+ designate differences in plaque and lysing characteristics; T6 and T4 have different host specificities), the new types of $T6r^+$ and $T4r$ are obtained among the progeny. The data of Delbrück & Bailey do not distinguish between induction of mutation in one phage by another multiplying in the same cell and recombination of virus elements. Hershey (78) has, however, reported experiments in which two viruses involved in mixed infection were strains of T2H differing in three characters and the results of these point to a recombination phenomenon of some sort. Thus, where one infecting virus was $hc^+ hb^+ r$ and the other $hc hb r^+$, where hc and hb refer to host spec-

ificity characters and r to lysis rate and plaque-type, seven phenotypes were recovered where eight would be predicted with free recombination.

Luria (79a) has recently made a preliminary report on his most remarkable discovery that two phage particles, each inactivated by ultraviolet radiation with respect to ability to multiply when absorbed by different sensitive bacterial cells, can somehow become reactivated when both are absorbed by the same cell. It is suggested by Luria that reactivation involves some type of recombination of gene-like loci that have undergone lethal mutation. X-ray inactivated phages cannot be reactivated in double or multiple infections.

These exciting results strongly suggest that bacterial viruses are structures containing at least several gene-like subunits. With the abundant experimental material available and the most elegant techniques developed by bacterial virus workers, the new field of virus genetics can be expected to develop rapidly.

CHEMICALLY INDUCED MUTATIONS

Nonspecific agents.—During the past twenty-five years or more numerous attempts have been made to induce gene mutations with various chemical agents. Almost all of these have either met with failure or the effects were so slight as to be of doubtful significance. The nucleus of higher plant and animal cells appears to be remarkably well insulated against changes in its chemical environment sufficient to modify genes. The first report of success came from the experiments of Auerbach & Robson, on *Drosophila melanogaster*, in which mustard oil was used. Later, when security regulations were relaxed, it was disclosed that mustard gas (β, β' -dichlorodiethyl sulfide) was much more effective (80). This was administered as a vapor. The incidence of sex-linked lethals was increased from 0.2 per cent in the controls to as high as 24 per cent in the treated series. Subsequent tests of related compounds showed that several of the nitrogen- or sulfur-mustard gases, e.g., β, β' -dichlorodiethyl-methylamine, were also active. Subsequent studies by Auerbach & Robson (81, 82, 83) have shown that nonlethal gene mutations, resulting in morphological characters, as well as chromosome aberrations are produced by mustard gases. Like those of x-rays the effects are nonspecific. Treatment of unfertilized eggs gave no increase in mutation frequency in paternal chromosomes subsequently introduced through the fertilizing sperm, suggesting that

the mustard gas effect is directly on the chromosomes. A similar conclusion has been drawn by Muller and by Timoféeff-Ressovsky regarding x-ray effects. It is not obvious how this result is to be reconciled with the observation of Stone, Wyss & Haas (73) that mutations are produced in bacteria by raying the culture medium and subsequently introducing the bacteria.

The effect of mustard gas on chromosome rearrangement has been confirmed by Koller working with the plant *Tradescantia* [cited by Auerbach *et al.* (83)]. Demerec (84) has found that lethal mutations are produced in *Drosophila*, while Horowitz *et al.* (85) have shown that biochemical mutations are produced in *Neurospora*. Tatum has concluded that the nitrogen mustard gas, β,β' -dichlorodiethylmethylamine, induces mutation in *Neurospora* (46) and in *Escherichia coli* (70), although the preliminary reports of this work give no data on mutation frequencies in untreated controls.

The somatic mutation theory of the origin of cancer cells has been considered many times. One of the objections to it has been the lack of mutation-producing effect of carcinogenic chemical substances. Demerec (84) has just recently reported that 1,2,5,6-dibenzanthracene, a known carcinogenic substance, applied to *Drosophila* as an aerosol of a solution in oil increases the incidence of sex-linked lethals by a factor of approximately twenty and that it also produces chromosome breaks. Strong (85a) had previously reported that the carcinogen methylcholanthrene induces coat color and other mutations in mice but because the rigorous genetic control that can be had in *Drosophila* is not possible in mice, there was some basis for skepticism regarding the mouse results. The results of experiments planned to correlate the mutagenic and carcinogenic activities of various compounds will be of great interest for they will provide important evidence for or against the somatic mutation theory of cancer.

Hadorn & Niggli (86) have reported that phenol induces mutations in *Drosophila* in excised ovaries treated with a dilute solution and implanted in untreated females. No confirmation of this reported mutagenic property of phenol has appeared to the reviewer's knowledge.

The experiments of Stone, Wyss & Haas in which mutations were induced in bacteria by x-irradiation of the culture medium have already been mentioned. It has not yet been reported whether the effect in this instance is specific or nonspecific.

Specific agents.—Interest in pneumococcus-transforming principle continues at a high level. McCarty & Avery (87, 88) have made further studies of these high polymers of desoxyribonucleic acid that in the presence of serum or serous fluids have the property of directing mutations of noncapsulated avirulent strains of pneumococcus to types specific with respect to the polysaccharide capsule. It has been shown that the transforming principle from Type III organisms is rapidly inactivated by minute amounts of purified desoxyribonuclease under conditions known to favor enzyme activity but not under those known to inhibit it. This evidence still further strengthens the argument that the transforming principle is the nucleic acid polymer and not an impurity. Transforming principles have been isolated from Types II and VI pneumococci and, like that for Type III, shown to be highly polymerized nucleic acids. A general review of the subject has been prepared by McCarty (89).

Whether the type-specific nucleic acids of pneumococci act by directing gene mutations, or are themselves taken up by the transformed organism to become a part of its gene system remains an unanswered question. In any event it seems clear that they do not serve merely as selection agents for mutations that would have occurred spontaneously. The fluid components have been investigated by McCarty, Taylor & Avery (90), who found that there are at least three of these, viz., the R antibody, a second protein component and a dializable factor.

Boivin, Delaunay, Vendrely & Lehault (91, 92, 93) have reported evidence favoring the view that mutations in *E. coli* from noncapsulated to capsulated forms can be directed with respect to capsular antigens by a desoxyribonucleic acid fraction. In this case serum factors are not necessary for the transformation to occur but it is essential that the bacteria be in a "susceptible" condition, not yet completely defined experimentally. The evidence that *Escherichia* transforming factors are nucleic acid is not as complete as that for pneumococcus.

Certain of the implications of the directed transformations in pneumococcus and *Escherichia* are obvious. A high degree of biological specificity, formerly thought to be limited to proteins and polysaccharides, can evidently be manifested by polymers of nucleic acid. Whether this specificity lies in chemical makeup of these molecules or in their physical configurations remains to be deter-

mined. As a result of this newly acquired knowledge we are certainly one step closer to an understanding of the gene and the mutation process.

CYTOPLASMIC INHERITANCE

There is a tremendous body of experimental evidence indicating that in higher plants and animals the stable hereditary mechanism consists of nuclear genes. On the other hand, every intelligent biologist has appreciated that cytoplasm is an essential medium through which genes act, that it has a certain amount of autonomy, and that it is of great importance in the process of differentiation. During recent years the interest of geneticists has been turned toward the cytoplasm by a number of instances of cytoplasmically inherited traits.

The fact that plastids arise only from pre-existing plastid or plastid primordia, and that mutation-like defects in these bodies, not reversible by nuclear genes, may occur and be transmitted for many cell and plant generations has been known for a long time. Rhoades (94) has recently reviewed our knowledge of the interrelation between higher plant plastids and nuclear genes.

The killer character in Paramecium.—Another interesting instance of cytoplasmic inheritance is that involving the so-called killer character in *Paramecium aurelia* studied by Sonneborn and his associates (95, 96). It is found that in a number of varieties of this protozoan there are two kinds of individuals, killers and sensitives. Killers produce a substance in the medium that kills individuals of a sensitive strain in a characteristic way. It has been shown that the killer character is dependent on a cytoplasmic component, called kappa, and a nuclear gene $+^k$. Kappa can only be multiplied if allele $+^k$ is present in the nuclei but it cannot be initiated by this allele; that is, some kappa must be present for more to be produced. If allele $+^k$ is replaced by k , an alternate form of the same gene, kappa is no longer multiplied. Preer (97) and Sonneborn (95, 96) have shown that in $+^k$ animals containing kappa, the multiplication of kappa need not keep pace with multiplication of the entire organism. Thus in variety 2 Preer finds that well-fed organisms kept under favorable conditions increase faster than kappa can multiply. By a series of ingenious experiments Preer was able to show that there are about 250 kappa particles in killer animals, that if this number is reduced below a threshold the animal be-

comes a sensitive that can revert to a killer if its division rate is reduced, and that if a high division rate is maintained for a sufficient time, some animals run out of kappa entirely and become permanent sensitives. He was able to show that an individual with only a single kappa particle is capable of reverting to a sensitive. Working with variety 4, Sonneborn has confirmed Preer's findings. Killers of this variety cannot be made to outgrow their kappa by optimal feeding but can be made to do so by growing them at a temperature of 38.5° C. If maintained for three to four days, this temperature is lethal, but if killers are exposed to it for shorter times, kappa is reduced in amount or eliminated. Thus after twelve hours killers become sensitives that can be reversed while after thirty-six hours they become permanently sensitive. Kappa can be transferred to $+^k +^k$ sensitive animal through the cytoplasmic bridge that may be formed during conjugation of the $+^k +^k$ sensitive (no kappa) animal with $+^k +^k$ (with kappa) animal. In this way Sonneborn has succeeded in transferring as few as two to four kappa particles. These leave the animal that received them sensitive but capable of becoming a killer when the fission rate drops at autogamy or during old age sufficiently to allow kappa multiplication to exceed cell fission by six or seven times—that is, when the number of kappa particles is increased to approximately 256 (29).

It is found by Sonneborn (95, 96) that all characters investigated in the B group of varieties, to which variety 4 belongs, involve a kappa-like cytoplasmic determinant. These characters include the killer character, antigenic properties and mating type. There is no evidence of comparable cytoplasmic units for similar characters in group A varieties.

Other instances of cytoplasmic inheritance.—Sonneborn (96) and others have found that resistance of a strain of *Paramecium aurelia* to antiserum directed against the same strain may be induced by treatment of the susceptible animals with the antiserum. Such resistance is known to be transmitted through many vegetative fissions, but not through sexual reproductions. Kimball (98) has observed that resistance is also induced by treatment with trypsin. It is supposed that susceptibility is dependent on an antigen which may be temporarily depleted through combination with antibodies or by tryptic digestion of the antigen itself or a precursor of it. Whatever is destroyed by trypsin is supposed by Kimball to be formed autocatalytically for it would otherwise be expected to

reappear after very few fissions. It is interesting to note that crosses between strains susceptible to antiserum against stock 60, variety 1, and others resistant to such an antiserum show the antigen concerned to be gene-controlled.

L'Heritier and his associates (99, 100) have for some years concerned themselves with the inheritance of susceptibility to carbon dioxide in *Drosophila melanogaster*. This trait is transmitted from generation to generation independently of chromosomes and apparently through the cytoplasm. It is much more effectively carried through the egg than through the sperm as would be expected if it were a cytoplasmic entity. Its sensitivity may be abolished temporarily or permanently by subjecting affected flies to either high or low temperatures and can be transmitted from an affected animal to a resistant one through ovary or brain transplants or through transfer of cell-free lymph [work cited by Ephrussi in discussion of paper by Sonneborn (95)]. The carbon dioxide-susceptibility character is apparently subject to an interpretation similar to that advanced for the killer character in *Paramecium* (100). Other instances of supposed cytoplasmic inheritance are those in wheat and oat rusts recently summarized by Johnson (101). These involve the transmission of pathogenic characteristics.

Mampell (102) has continued his studies on a strain of *Drosophila pseudoobscura* showing a high spontaneous mutation rate. He postulates the existence of both a mutator gene and a cytoplasmic component that can be transferred by contact from one strain to another or even from *Drosophila pseudoobscura* to *Drosophila melanogaster*. The situation is not yet well understood and the evidence for the self-reproducing cytoplasmic factor postulated by Mampell is not entirely convincing.

It is, I believe, cause for reflection that none of the instances in which cytoplasmic inheritance is firmly established is a self-duplicating gene-like structure involved that cannot reasonably be supposed to be gene-dependent. Thus, while Sonneborn's kappa is certainly self-duplicating in the sense that some must be present before more can be formed, it cannot continue to be elaborated in the absence of the $+^k$ allele of a specific gene. It is true that such gene-dependence has not always been demonstrated, but as Sonneborn (96) has pointed out this may mean merely that the active forms of all essential genes are present in all strains investigated. An alleged case of cytoplasmic transmission that would seem to the

reviewer to require a basic change in our conceptions of genic control is that first reported by Lindegren, Spiegelman & Lindegren (103, 104) in yeasts. Here, it is claimed, there exists an enzyme melibiase that in the presence of the substrate melibiose is capable of indefinite self-duplication in the absence of the gene that initiates it. It is true that the supposed self-duplicating cytoplasmic entity may be an enzyme precursor rather than the enzyme itself but it remains that we have here a postulated cytoplasmic unit that is capable of permanently substituting for a gene. There has been a great deal of discussion of this situation (105). In fact an elaborate theory (the so-called cytogene theory) has been developed as a result of the reported facts (106, 107). It appears from a recent publication of Lindegren & Lindegren (107) that the critical experiments on which the self-duplicating enzyme hypothesis was based cannot be repeated. The cytogene theory has apparently been abandoned (45). Evidently a final appraisal of the situation in yeast must await the straightening out of the basic experimental facts.

Symbiosis and the origin of cytoplasmic hereditary units.—Altenburg (108) has suggested that cytoplasmic factors like kappa in *Paramecium* might well have arisen from symbionts such as the green algae found in *Paramecium bursaria*. While, as Sonneborn (96) has pointed out, strong arguments can be advanced against such an interpretation in the case of the killer character, it is not easy to rule out the symbiont hypothesis completely. In fact, on logical grounds one would expect in certain cases the genes of an intracellular symbiont to take over the functions of one or more genes of the host organism. This has apparently occurred in the cockroaches where bacteria-like organisms live symbiotically in the fat body cells of the insect. The "bacteroids" have not been cultivated in the absence of living cells nor can the host live if the symbionts are abolished by penicillin treatment (109). Apparently each of the symbiotic partners has become irreversibly dependent, presumably through a process of complementary gene mutation. The cockroach is dependent on genes of the bacteria and vice versa. The degenerate bacteria are transmitted cytoplasmically through the egg of the insect. If the intracellular symbiont were to regress somewhat further, affect the host in a nonvital manner and to be less obviously descended from a bacterium, one would certainly conclude that it constituted a self-duplicating cytoplasmic hereditary factor.

How general is cytoplasmic inheritance?—However gene-like cytoplasmic hereditary units may arise, it does not seem likely that they are of widespread occurrence. If self-duplicating enzymes or enzyme precursors of the Spiegelman-Lindgren variety were the rule we would not expect experimentally to find the numerous biochemical mutants that have been demonstrated in *Neurospora* and *Ophiostoma* and which differ in nuclear genes from their synthetically more complete counterparts. Furthermore we should expect to find many deficiency mutants that differ from the original type only in a cytoplasmic component. It seems highly probable, as Sonneborn (96) and others have pointed out, that cytoplasmic substitutes for nuclear genes would become established only under special circumstances. The ciliates in which a division of labor between two kinds of nuclei has become established might well constitute just such a special case. The almost universal distribution among existing organisms of the nuclear gene mechanism of heredity is good evidence that it represents the most effective machinery that mutation and natural selection have so far succeeded in devising.

Plasmagenes in another sense.—It seems entirely possible that many nuclear genes may direct the synthesis of elements capable of multiplication in the cytoplasm. As Wright (110), Burnet (111), Spiegelman (104), Boivin & Vendrely (112), and others have pointed out, this assumption is not unreasonable on the basis of our knowledge of phenomena such as cellular differentiation and the multiplication of adaptive enzymes. Wright (110) has indicated how such gene-initiated self-duplicating cytoplasmic elements, which have been called plasmagenes, might, under the influence of local environmental conditions in a developing organism, become systematically modified without losing the ability to multiply. An orderly succession of such modifications might well underlie the process of differentiation. Plasmagenes in this sense are not cytoplasmic substitutes for nuclear genes but rather are complementary to them.

EVOLUTIONARY CONSIDERATIONS

The Horowitz hypothesis (113) which has to do with the evolutionary history of systems of biosynthetic reactions was mentioned in a previous volume of this series (2). There are two recent contributions that have a bearing on this general question. One is the observation by Monod (114) that a methionine-requiring

Aerobacter mutant grows appreciably faster on a methionine-containing medium than does the wild type strain from which it was derived. One cannot, of course, generalize from a single instance of this kind, but it would be most significant from the standpoint of evolution if it were frequently the case that loss mutants were more successful than their synthetically more complete counterparts in an environment rich in the product of the reaction blocked in the mutant. It is of course a well known fact, as has been pointed out especially by Lwoff (115) and by Knight (115a), that evolutionary specialization through loss of synthetic ability has occurred in many groups of organisms. It seems probable that genes that become inactive in this process are available for mutations that add new synthetic reactions and thus serve to make possible positive evolution (116).

Houlahan & Mitchell (117) have reported a situation in *Neurospora* that may have an important bearing on the question of positive evolution. It is found that the pyrimidine requirement of a mutant strain can be abolished through mutation of a gene that is genetically independent of the one involved in the original mutant change from wild type. Such "suppressors" have been known for a number of mutant traits in *Drosophila* but this is one of the clearest so far as knowing the affected system of synthesis is concerned. If the normal function of one gene can be taken over by a mutant form of another, it seems possible that the direction of an entirely new reaction could be taken over by an unneeded gene in essentially the same manner.

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